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SERUM ALKALINE PHOSPHATASE RESPONSE TO THE ADMINISTRATION OF CORTISONE IN RATS[1]

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CORTISONE administration was noted to have a variable effect on rat serum alkaline phosphatase (1, 2). Alkaline phosphatase in rachitic rats fell when the rats received cortisone (1). A fall in alkaline phosphatase was also demonstrable after cortisone administration in normal rats. However, some of the animals in the same group had marked elevations of serum alkaline phosphatase (2). Winter *et al.* reported an increase in alkaline phosphatase in rats given cortisone (3). Cortisone has also been reported to increase the activity of some of the alkaline phosphatases in the liver (4, 5).

One infant with hypoglycemia, glycosuria and depressed serum alkaline phosphatase was given cortisone for the hypoglycemia. Coincident with the rise in blood glucose the serum alkaline phosphatase rose to normal levels (6). Fraser and Laidlaw reported a similar elevation of serum alkaline phosphatase in a child with hypophosphatasia who was treated with cortisone (7). On the other hand patients treated with cortisone for a variety of reasons have been found to have depressed serum alkaline phosphatase acitvity (8, 9). The present study was an attempt to clarify the effect of cortisone on serum alkaline phosphatase activity.

MATERIALS AND METHODS

White male rats weighing between 150 and 225 gm. were used. Group I animals had blood withdrawn by cardiac puncture at the beginning and end of a three day period in which they received laboratory chow and water *ad lib.*

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Group II rats were fasted from 1 to 3 days but given water *ad lib.* Blood was drawn from the heart for alkaline phosphatase determination at the start and end of the study period in the fasted animals and in the comparable controls. Body weights were recorded at the same time.

Group III animals all received cortisone intraperitoneally in daily doses of 2 to 13 mg. per 100 gm. of body weight. They had free access to rat chow and water. Blood was drawn by cardiac puncture before treatment was begun and serum alkaline phosphatase measured. The rats were weighed at this time and then again after 3 days of cortisone treatment when blood was once more drawn in the same manner.

Originally the experiment was carried out with two groups of 12 and 24 animals who were treated as groups II and III except that blood was withdrawn only at the end of the experiment and compared with 6 untreated controls in each group. The findings were the same in these animals which were less well controlled as in the other groups and will not be considered further.

Serum alkaline phosphatase was measured in Bodansky units (10).

RESULTS

In Group I there was no significant change in serum alkaline phosphatase measurements in control animals from which blood wss obtained initially and at the end of the 3-day period. These animals gained less weight than untreated control animals presumably as a result of the blood loss and attendant trauma.

Table 1 relates the change in body weight of the animals to the change in serum alkaline phosphatase. The values for the deviations are expressed as per cent change from the original value. The individual doses of cortisone and the significances of the differences between the groups of treated and untreated animals are also given.

From these figures it is seen that there was no significant change in serum alkaline phosphatase in the untreated animals that gained weight. However, with increasing loss of weight as a result of starvation and withdrawal of blood there was an increasing depression of the serum alkaline phosphatase. The serum alkaline phosphatase change correlated better with the change in weight than with the actual dosage of cortisone in the Group III animals. The larger doses of cortisone which more consistently produced larger depressions in weight were associated with a fall in serum alkaline phosphatase. The smaller doses of cortisone which more often were associated with a gain in weight or a minimal weight loss led to an increase in the serum alkaline phosphatase values. When the animals in both studies are considered in groups according to weight change, i.e., those which gained over 2% of their body weight, those which lost less than 5% of their body weight, those which lost 5 to 16% of their body weight, it is apparent that the animals which received cortisone have higher serum alkaline phosphatase values in each weight group. Although the larger doses of cortisone caused a fall in serum alkaline phosphatase, this fall was less than that found in those animals which had lost the same amount of weight when starved but did not receive cortisone. When the animals receiving

TABLE 1. CHANGE IN SERUM ALKALINE PHOSPHATASE IN RELATION TO WEIGHT CHANGE IN CONTROL AND CORTISONE TREATED ANIMALS

Control animals (I and II)			Experimental animals (III)			
Rx	Weight change % of initial weight	Ser. alk. phosphatase change % of initial value	Signif. of difference between means	Ser. alk. phosphatase change % of initial value	Weight change % of initial weight	Rx mg. of cortisone per 100 gm./day
Fed	+ 4.0	−2		+31	+ 5.4	3.4
Fed	+ 3.7	+7		+190	+ 5.2	6.5
	+ 3.9	+3		+110	+ 5.3	
Fed	− 0.4	−18		+37	+ 1.8	2.2
Fasted	− 3.6	−33		+16	+ 0.6	2.8
Fasted	− 4.0	−48		+32	+ 0.4	2.3
				−39	− 0.9	2.3
	− 2.8	−33 ± 8.7*		− 8	− 1.1	2.6
				−15	− 1.9	4.6
			Phosphatase P < .05	+ 3	− 3.2	4.6
				−14	− 3.8	4.7
				+65	− 3.9	2.8
				− 2	− 4.1	5.1
				−19	− 4.5	12.9
				+ 5	− 4.7	5.8
				+5 ± 8.2*	− 2.0	
Fasted	− 6.3	−48		+44	− 5.5	6.0
Fasted	− 8.4	−27		−62	− 5.5	12.3
Fasted	− 9.2	−73	Phosphatase	+30	− 6.5	10.0
Fasted	−10.6	−76	P < .02	−17	− 6.7	9.6
Fasted	−12.3	−70		−83	− 9.1	11.3
Fasted	−13.0	−74	Weight	−28	−10.7	11.9
Fasted	−13.7	−62	P > .5	−26	−12.1	10.0
Fasted	−15.2	−85		−22	−12.5	3.1
				−16	−16.0	3.5
	−11.1 ± 1.1*	−64 ± 6.6*		−20 ± 13*	− 9.4 ± 1.2*	
Fasted	−16.9	−62				
Fasted	−18.8	−83				
Fasted	−19.3	−94				
Fasted	−20.4	−77				
Fasted	−21.2	−87				
Fasted	−21.9	−72				
Fasted	−22.7	−87				
Fasted	−23.8	−67				
Fasted	−24.0	−82				
	−21	−79				

* SEM.

cortisone gained weight the serum alkaline phosphatase rose dramatically. The mean depression of serum alkaline phosphatase was less in the cortisone-treated animals which lost less than 5% of their body weight than it was in the fasted animals with equal weight loss at the 95% level of confidence. Similarly amongst the animals which lost from 5 to 16% of their initial weight the cortisone-treated animals had a smaller depression of serum alkaline phosphatase that was significant at the 98% level.

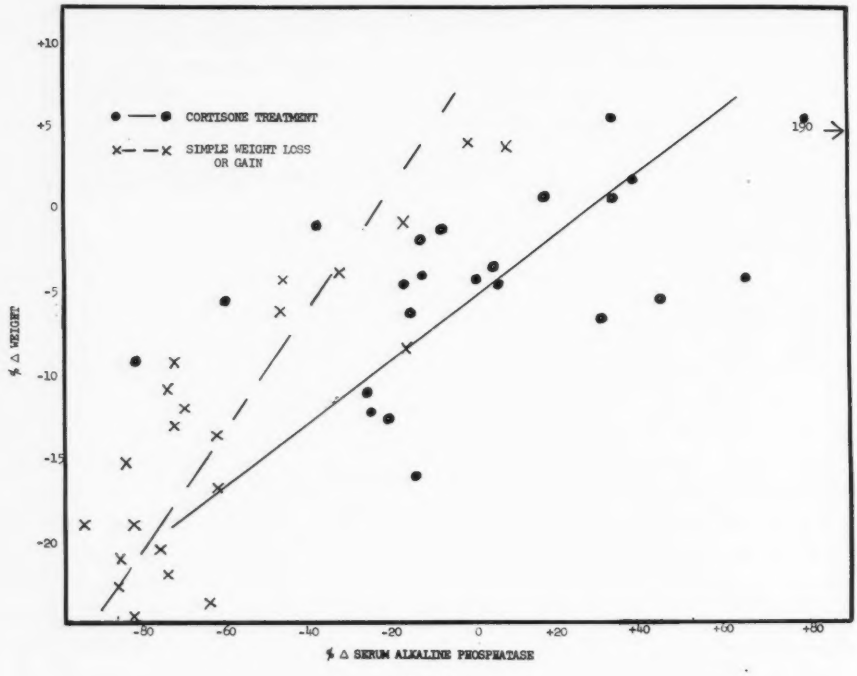

FIG. 1

Figure 1 graphically portrays the data. Serum alkaline phosphatase is plotted against change in weight. The calculated regression lines for the animals that received cortisone ($S = 5.4\ Y + 27.6$, $r = .54$ $p < .02$) and for the animals that did not receive cortisone ($X = 2.73\ Y - 25.3$, $r = .83$ $p < .01$) are drawn in.

DISCUSSION

As noted previously, the administration of cortisone led to variable changes in the level of serum alkaline phosphatase in the rat. From the results described, we have assumed that the direct action of cortisone is to cause an increase in serum alkaline phosphatase. As the dose of cortisone is increased it causes weight loss. Accompanying the weight loss there is a secondary depression of serum alkaline phosphatase. However, the depression of serum alkaline phosphatase associated with weight loss and the administration of cortisone was not as great as that found in animals with similar weight losses (up to 16% of body weight) from starvation alone. Thus it would seem that cortisone always has a direct effect tending to elevate serum alkaline phosphatase but that this action may be obscured by the depressing effect of weight loss which accompanies the administration of large doses of cortisone.

SUMMARY

Rats given cortisone in increasing doses demonstrated an elevation of serum alkaline phosphatase until the dose of cortisone was large enough to produce a weight loss. With increasing weight loss associated with cortisone treatment the serum alkaline phosphatase dropped progressively. However, the depression of serum alkaline phosphatase following weight loss from cortisone administration was not as great as that following weight loss from starvation.

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EFFECTS OF A BACTERIAL POLYSACCHARIDE (PIROMEN) ON THE PITUITARY-ADRENAL AXIS: FURTHER ASPECTS OF HYPOPHYSEAL-MEDIATED CONTROL OF RESPONSE

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THE bacterial polysaccharide complex, Piromen®, has afforded clinical relief in a number of allergic conditions (1–5). Part of this therapeutic usefulness may be credited to a capacity for activating the pituitary axis and increasing adrenocortical secretion (6–10).

From the standpoint of metabolism, the pattern of stimulation of the pituitary-adrenal system by this preparation has been shown to have certain individual characteristics (11). For example, the significant positive nitrogen balance which follows intravenous injection of larger amounts (100 μg.) of Piromen is not easily reconcilable with the more typical cortical-metabolic sequelae which also accompany it (eosinopenia, increased corticoid excretion). Similar changes in the endocrine system have been observed in the animal; the response, reflected in the pattern of adrenal ascorbic acid, cholesterol content and cellular alterations in the adrenal cortex of the rat, has been described in an earlier report (12). Some aspects in these indices of cortical stimulation resembled those which follow the administration of ACTH, but there remained certain different features in the total picture. For example, the interval of adrenal ascorbic acid and cholesterol depletion was prolonged and more intense (12) yet there was no delay in the repletion phase for these same constituents. Definite qualitative and quantitative variations in pituitary-adrenal response occurred as dosage levels of Piromen were increased or decreased. Additional increments, each 5 μg. per 100 gm. of body weight, brought progressive depletion in adrenal ascorbic acid and cholesterol but no alterations in the histologic configuration appeared until the threshold value (25 μg.) was reached. Thymotroph-

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[1] Piromen® is a sterile, nonprotein and non-anaphylactogenic bacterial polysaccharide complex. It is prepared as a stable suspension in $\frac{1}{6}$ *M* sodium r-lactate containing 1:10,000 parts of thiomerosal. Piromen is a product of Travenol Laboratories, Inc., a subsidiary of Baxter Laboratories, Inc., Morton Grove, Illinois.

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ism and somatotrophism were observed after administration of this polysaccharide despite frank evidence that the drug causes active cortical discharge (11), an event usually associated with involution of the thymus, cessation of body growth and actual loss in body weight (13).

It is reasonable to assume that these effects by Piromen are mediated by the pituitary gland. The adrenals of the hypophysectomized rat are not depleted of ascorbic acid or cholesterol stores after administration of this preparation (12). Two possible explanations for the mechanisms involved in this hypophyseal stimulation have been considered. Piromen may stimulate the hypophysis thus releasing adrenocorticotrophic hormone alone or in company with other trophic (perhaps somatotrophic) secretion. We have focused attention on these effects in both the intact and the hypophysectomized rat. The purpose of this communication is to present the results of experiments designed to test these hypotheses.

METHODS

Animals[3] were selected, housed and fed according to procedures previously outlined (12). One-third unit of ACTH (USP) in gel was allowed for each 100 gm. of body weight, given twice daily subcutaneously. Piromen was administered in a single daily intraperitoneal injection of 1.0 or 10 μg. per 100 gm. of body weight. Animals reserved for comparison studies (controls) were placed in two equal groups; members of one group were given $\frac{1}{6}$ *M* sodium r-lactate (0.25 ml.) intraperitoneally while animals in the second group received 0.25 ml. of 16% gelatin solution, subcutaneously.

Animals of the same sex and weight were selected for hypophysectomy and an interval of 24 hours was allowed for recovery from operative stress. On the basis of 100 gm. of body weight either 1.0 or 5.0 μg. of Piromen were given once daily by the peritoneal route. ACTH, $\frac{1}{3}$ unit, was injected subcutaneously twice daily for others. Animals used in control studies were given either sodium r-lactate or 16% gelatin solution.

Intact and hypophysectomized animals in both treated and control groups were sacrificed by decapitation 24 hours after the 21st treatment-day. At autopsy the sella turcica was examined to determine the completeness of hypophysectomy; animals with residual pituitary tissue were discarded.

Throughout the experiment careful record was kept of changes in body weight as an index of anabolic or catabolic trends. For gravimetric analyses, thyroid, spleen, kidneys, testes and adrenal glands were removed, carefully freed from extraneous tissue and weighed on a Roller-Smith torsion balance. The right adrenal gland was prepared for histologic analysis by fixation in 10% buffered neutral formalin (Lillie); sections were then frozen, cut to 6 μ thickness and stained for lipid content with either sudan orange (hematoxylin) or sudan black B (carmalum counterstain). Remaining tissues were fixed in the formalin solution, dehydrated, embedded in paraffin, cut to the same thickness, and stained with hematoxylin and eosin.

For quantitative chemical determinations of adrenal ascorbic acid the left adrenal gland was removed quickly, carefully trimmed free of fat, weighed to the closest milligram fraction (0.2 mg.) and homogenized in 2.5% metaphosphoric acid. Ascorbic acid content was then determined by the method of Mindler and Butler (14), using the Coleman spectrophotometer.

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[3] In order to maintain clarity the number of animals used in each test group is given in the proper Table or Figure.

RESULTS

Intact animals

Body Weight: Animals given ACTH for 21 days showed retardation in their normal rate of growth (Table 1) and weighed 25 gm. less, on the average, than those animals given Piromen or left untreated. For the last-named groups there was no evidence of growth inhibition and the rates of growth were approximately the same. The anti-anabolic or catabolic effects of ACTH appeared very shortly after the beginning of treatment.

Thyroid: The thyroid gland increased slightly in weight in animals treated with Piromen and with ACTH (Table 1). While not dissimilar on a

TABLE 1. COMPARISON OF THE EFFECTS OF PIROMEN AND ACTH ON THE HYPOPHYSECTOMIZED SPRAGUE-DAWLEY RAT AFTER 21 DAYS OF TREATMENT*

Treatment	Number of animals	Body weight (gm.)			Thyroid (mg.)	Thymus (mg.)	Spleen (mg.)	Testis (mg.)	Kidney (mg.)	Adrenal (mg.)	Mg. asc. acid/100 gm. adrenal
		Initial	Final	Gain or loss							
Intact animals											
Control	16	104	186	+82	12.9	429	561	1174	747	16.0	414
1 μg. Piromen	16	109	192	+83	15.7	432	574	1257	765	17.1	410
5 μg. Piromen	16	106	185	+79	14.0	439	589	1151	783	18.3	430
10 μg. Piromen	16	114	199	+85	16.4	442	580	1469	811	20.1	436
½ U ACTH	16	113	173	+60	14.1	137	408	1105	727	32.9	498
Hypophysectomized animals											
Control	13 (15)†	112	98	−14	6.2	163	176	144	339	5.3	229
1 μg. Piromen	8 (12)	100	92	− 8	5.8	162	215	110	330	5.2	215
5 μg. Piromen	6 (12)	100	89	−11	5.6	155	208	108	366	4.6	252
10 μg. Piromen	8 (12)	116	102	−14	6.3	198	178	171	377	4.9	218
½ U ACTH	6 (12)	106	70	−36	5.6	14	110	104	366	18.2	227

* The values given are the average, actual weights. In the main, statistical analyses of these values, using ratios o organ weight to initial body weight or to final body weight, or to increment of change, showed that the differences were highly significant.

† Figures in parentheses indicate the number of animals at the beginning of the experiment.

gravimetric basis, there were marked histologic differences between thyroid glands removed from Piromen-, and ACTH-treated animals. Thyroid follicular epithelium was flattened and there were accumulations of interfollicular connective tissue and foci of atresia after ACTH. By contrast, thyroid tissue was normal after Piromen.

Thymus: In animals given Piromen the thymus was not only maintained but a parallel thymotrophic effect could be seen as the dosage increased; this could be confirmed gravimetrically and by histologic observation. Dramatic thymic involution followed the administration of ACTH (Table 1); parenchymatous elements were almost obliterated leaving only the stromal endoskeleton of reticular connective tissue.

Spleen: The companion reticuloendothelial organ, the spleen, was altered by ACTH with splenic involution and dissolution of the white cell elements. Spleen tissue pulp substance was well-maintained by Piromen (Table I).

Testes: Significantly heavier testicular weight was found in animals given

Piromen at the 10 μg. dose level compared to animals in any other group. The gonadotrophic effect noted at 1.0 and 10 μg. dosage levels was absent at the intermediate (5.0 μg.) level. Testes taken from animals given ACTH were lightest in weight. However, there were no detectable histologic differences.

Kidney: The kidney increased in weight as the dosage level for Piromen was raised. Histologically, these glands were normal. Again, this organ weighed less in animals given ACTH. Microscopically, kidney tissue removed from members of the latter group showed either fibrosed glomeruli

FIG. 1. Kidney. Intact male rat given $\frac{1}{3}$ unit ACTH in gel/100 gm. (s.c.) twice daily for 21 days. Note shrinkage of glomeruli and colloid-filled tubules. H & E, 6μ, ×100.

or hyperplastic proliferation of the capillary tuft. In some instances glomeruli were completely shrunken within their capsule. The tubules were filled with colloid material which stained bright red with eosin. Epithelial cells in the lining were frequently vacuolated and there were multiple foci of interstitial leukocytic infiltration. Viewed *in toto* the microscopic picture resembled strongly that found in renal injury of the arteriolar glomerulosclerotic type (Fig. 1).

Adrenal: Piromen induced a moderate but sustained increase in adrenal weight and this increase could be correlated with the dose level. A similar trophic action on adrenal ascorbic acid levels was observed. The most dynamic increases in adrenal weight and ascorbic acid levels followed treatment with ACTH; histologic examination confirmed the existence of severe and unphysiologic stimulation. The cortices were massively hypertrophied, the cells vesiculated and packed with refractile lipid bodies. The capillaries

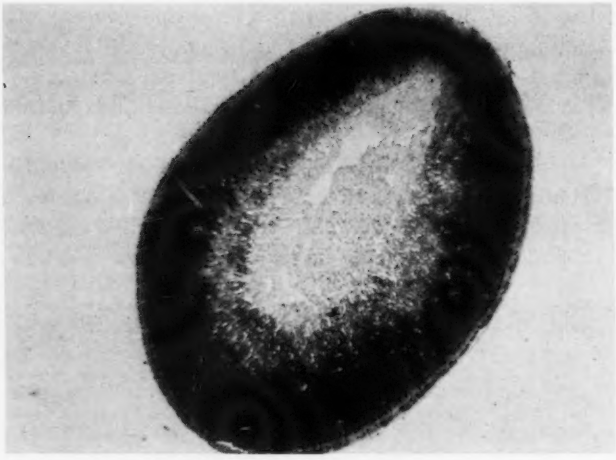

FIG. 2. Adrenal. Intact male rat given 1 μg. Piromen/100 gm. (i.p.) once daily for 21 days. Some cortical discharge has occurred, as indicated by the appearance of a lipid-free zone between the zona glomerulosa and outer fasciculata, with lipid depletion in the zona reticularis. Sudan Orange, 5μ, ×27.

of the zona reticularis network were distinctly dilated (cf. Fig. 5 with Figs. 2, 3 and 4). Only conditions of severe stress approaching adrenal cortical exhaustion produce this histologic picture (15).

By contrast hyperplasia and hypertrophy of a more "benign" type were seen in the adrenal glands of animals given Piromen. Lipids were only

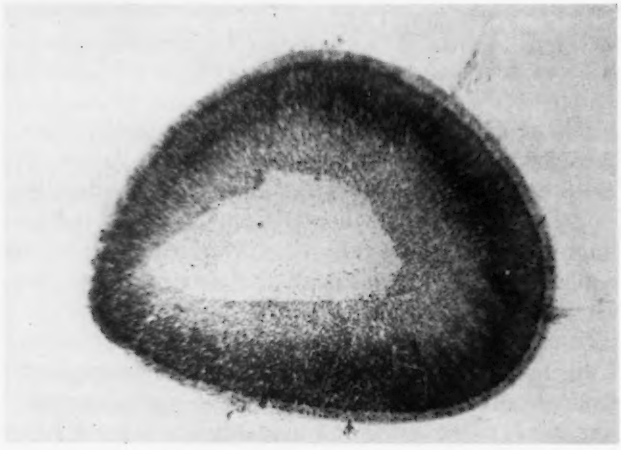

FIG. 3. Adrenal. Animal given 5 μg. of Piromen. Note increased depletion from zona reticularis and inner fasciculata indicating a higher level of activity. Sudan Orange, 5μ, ×27.

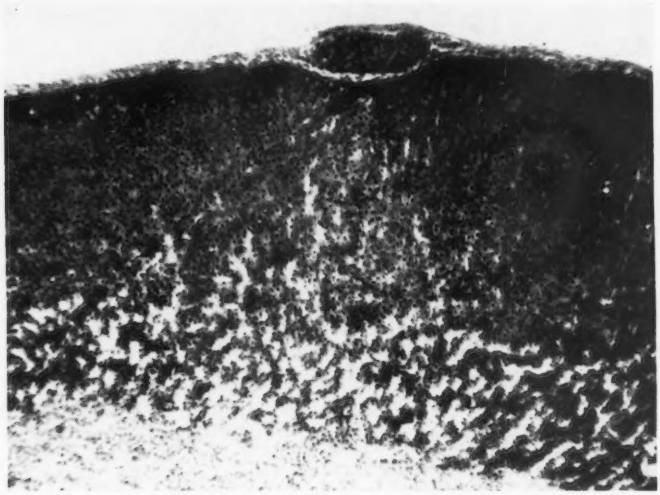

FIG. 4. Adrenal. Animal given 10 μg. Piromen. Gland is hypertrophied. Note intracapsular nodule of growth and distinct cell boundaries. The depletion of lipid from the zona reticularis and inner fasciculata is not apparent here. Sudan Orange, 10μ, $\times$100.

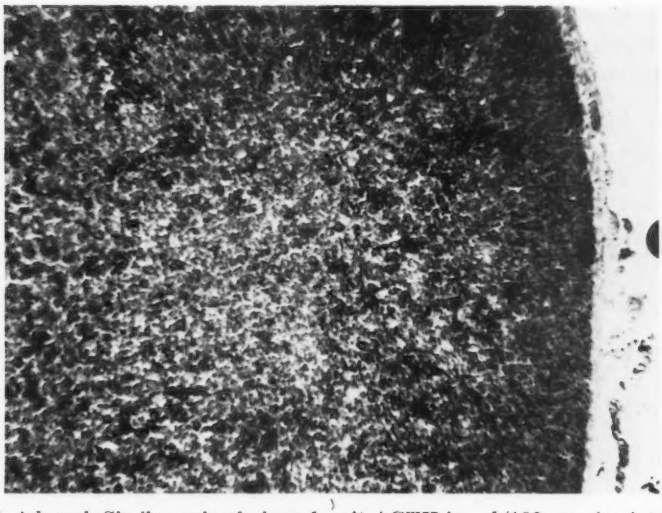

FIG. 5. Adrenal. Similar animal given $\frac{1}{3}$ unit ACTH in gel/100 gm. (s.c.) twice daily for 21 days. Photo taken at same magnification as Fig. 4. Note hypertrophy of cortex. Growth is so massive that portions of the inner fasciculata and zona reticularis are not included. Cells are vacuolated. The refractile lipid bodies are seen as irregular white areas in this photo. Sudan Orange, 10μ, $\times$100.

moderately depleted during the period of treatment; this depletion was more pronounced in the zona reticularis in animals given 5 μg. than in those given smaller (1 μg.) amounts. Greater growth-stimulating activity, associated in some instances with the appearance of intracapsular cortical nodules (Fig. 4), was induced by 10 μg. of Piromen. The zona glomerulosa and outer fasciculata were not depleted of lipids. Normal staining affinities persisted (sudanophilia). The zona reticularis and inner fasciculata showed active lipid depletion, while the cortices were overtly but not overwhelmingly stimulated.

Hypophysectomized animals

All animals lost weight after removal of the hypophysis. Little difference could be found between Piromen-treated and control rats. The weight loss after ACTH was three times that seen in animals given Piromen and two and one-half times that found in animals in the untreated groups (Table 1).

Deprivation of thyrotrophic hormone reduced thyroidal activity in all hypophysectomized animals. The thymus was shrunken in appearance and weighed less after hypophysectomy. The thymotrophic effect attributed to Piromen in the intact rat was absent. Very pronounced thymic involution followed the use of ACTH; this involution was far greater in degree than that seen in the intact rat after the same treatment. Degeneration was so complete that practically no thymocytic elements remained (Fig. 6). Splenic involution followed the administration of ACTH as it did in the intact rat and reduction in red and white pulp composition was equally severe.

FIG. 6. Thymus. Hypophysectomized male rat given ⅓ unit ACTH in gel/100 gm. (s.c.) twice daily for 21 days. All thymocytic elements have been obliterated and only a connective tissue endoskeleton remains. H & E, 6μ, ×26.

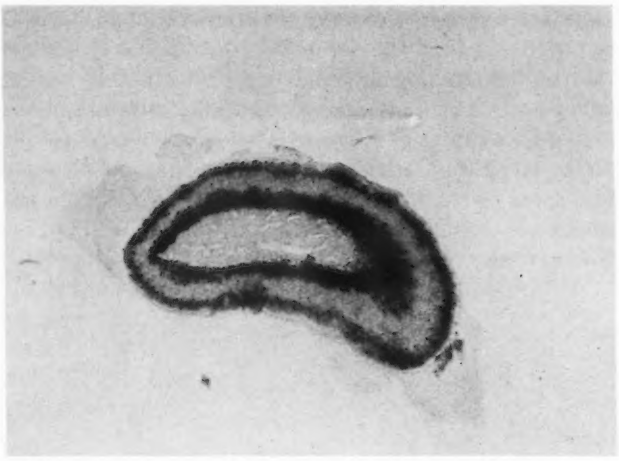

FIG. 7. Adrenal. Hypophysectomized male rat given 5 μg. of Piromen. No evidence of adrenal maintenance or repair. Intact zona glomerulosa, wide lipid-free zone, and atrophic zonae fasciculata and reticularis, are typical of the adrenal post-hypophysectomy. Sudan Orange, 10μ, ×26.

Weight and cell alterations in testes and kidneys were those considered typical of the post-hypophysectomy state (16). Piromen did not exert the same renotrophic effect which it exhibited in the intact animal. Few differences existed between the groups regardless of the treatment used.

Adrenals, whether removed from Piromen-treated or untreated animals,

FIG. 8. Adrenal. Same treatment as Fig. 7. Higher magnification shows the well-maintained zona glomerulosa, wide lipid-free zone and atrophic fasciculate portions. Sudan Orange, 10μ, ×100.

weighed less after hypophysectomy. On a gravimetric basis, ACTH assisted in the maintenance of the adrenal weight (Table 1) but such medication did not restore the normal lipid complement. The zona reticularis and both inner and outer portions of the zona fasciculata were depleted of their sudanophilic material (Fig. 9). The ascorbic acid content of these glands did not exceed that found in Piromen-treated and control animals. Again Piromen was found to be without trophic effect when the pituitary gland was removed. Adrenals from Piromen-treated rats had broad, lipid-free zones and atrophy of the zona fasciculata, the typical histopathologic aftermath of hypophysectomy (Figs. 7 and 8).

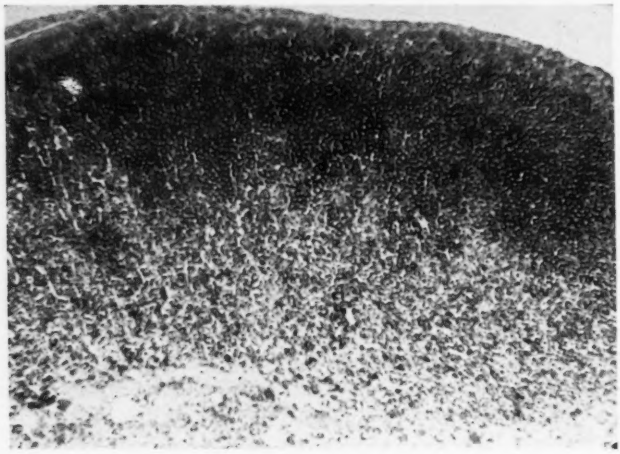

FIG. 9. Adrenal. After $\frac{1}{3}$ unit ACTH. Gravimetrically, the adrenal is well-maintained. However, note coarse physical appearance of lipid and extensive depletion (or lack of lipid restoration) of lipid in all inner zones. Sudan Orange, 10μ, $\times$100.

DISCUSSION

The essential role of the hypophysis in the endocrinal response to a bacterial polysaccharide preparation is again demonstrated. In addition, data presented strengthen the concept, previously introduced (12), of Piromen as a preparation producing effects beyond those to be expected of a "nonspecific stress stimulus" (11) causing endogenous release of ACTH. Piromen does cause alterations in adrenal ascorbic acid, cholesterol and in the cellular pattern and these are similar to but not identical with those evoked by ACTH (12). While the amount of ACTH released may be regulated by the dose-level of Piromen employed (11, 12) not all physiologic phenomena can be explained on the basis of endogenous ACTH release alone. Piromen can, for example, increase adrenocortical activity without the usual negative nitrogen balance. As shown graphically in Table 1 Piromen does not interrupt the normal growth rate pattern of the rat. After administration of ACTH catabolic or anti-anabolic sequelae could be traced.

These observations are in harmony with the clinical findings of Conn and co-workers (11). These investigators reported recently that Piromen induced cortical activity in patients which was measurable in terms of eosinopoenia and steroid excretion. At the same time "significant positive nitrogen balance as well as retention of both potassium and sodium . . . " could be confirmed. Another unusual aspect of this metabolic response to Piromen lay in the absence of the initial rise in urinary uric acid. The positive nitrogen balance observed in both human subjects and animals in association with retention of sodium and potassium is usually seen after the administration of growth or somatotrophic hormone (STH). In view of these known trophic effects of Piromen and STH on the reticuloendothelial system, one is tempted to postulate, at least temporarily, that Piromen may cause simultaneous release of ACTH and STH with or without other trophic hormones.

When one relates the responses obtained with Piromen with the dose-increment it would seem that in the rat a hypophyseal threshold exists for this drug.[4] If gravimetric data on body weight, thyroid, and testicular weight in the intact animals be used as indices of somatotrophism, the changes produced by 5 μg. of Piromen and 1/3 unit of ACTH are quite similar; for example, the anti-anabolic effects. Adrenocorticotrophic activity increases when 10 gammas are administered, but definite trophic changes appear in other glandular elements. Other indications of a pituitary threshold for Piromen has been cited (12). It was found that increasing dosage levels, to 25 μg. per 100 gm. of body weight, will induce profound changes in adrenal ascorbic acid and cholesterol in the rat: these changes are not accompanied by the histologic alterations in adrenal tissue. Only at 25 μg. levels or above are such alterations to be seen. These results suggest a clue to what may be a basic mechanism, namely, that Piromen may provide monotrophic or pan-hypophyseal stimulation, depending on the dosage employed. Obviously a special study would be required to test this hypothesis.

The thymotrophic or splenotrophic effects of Piromen are quite interesting. Piromen causes granulocytopoenia and agranulocytopoenia, followed by granulocytosis and increased numbers of agranular cells (17, 18). While both ACTH and Piromen cause lysis of white blood cells, the latter behaved in a dichotomous manner. ACTH brought severe thymic and splenic involution with white blood cell lysis; Piromen either (a) produced lysis initially, followed by very rapid cell replacement after an intense hematopoietic stimulation, or (b) directly protected or supported elementf os the

[4] Conn (11) found patients showed no measurable adrenal response when 5 to 10 μg. of Piromen were given; after the dose level was raised to 25 μg., eosinopenia occurred unattended by increased corticoid output. When doses of 50 to 100 μg. were given they elicited the usual spectrum of metabolic changes indicative of increased adrenocortical activity.

reticulo-endothelial system. A heightened stimulation of blood-forming tissues occurs in the rabbit after prolonged Piromen administration, and this stimulation extends to the extra-medullary tissues of the liver and spleen (19). This enhancement of granulocytic activity has been the basis in part for the clinical application of the preparation in the treatment of allergic conditions (1–5).

Renotrophic effects in the intact animal have been frequently observed in our laboratories (6). The renal artery is markedly distended following treatment with Piromen which may explain these results. Arteriolar glomerulosclerotic lesions in the kidneys of animals given ACTH may have been induced due to the "conditioning" caused by an excessive secretion of DOC-like steroids (20, 21).

SUMMARY

The bacterial polysaccharide complex, Piromen, stimulates increased adrenal cortical activity attended by definite adrenocorticotrophic events. This hypophyseal-mediated response is not accompanied by the usual anti-anabolic changes associated with the corticotropic and corticotrophic effects of ACTH—body weight loss and involution of reticuloendothelial elements.

It is postulated that this preparation stimulates the pituitary gland releasing ACTH and other anterior pituitary trophic hormones such as the growth hormone. The end effect of this dual release is a modification of the usual metabolic response to ACTH by the growth hormone.

A pituitary threshold exists which is related to the dose of Piromen and the degree of pituitary-adrenal response.

Finally, the data demonstrate that the endocrinal response to Piromen, in the rat, is dependent on the presence of the hypophysis.

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THE INFLUENCE OF THE PITUITARY GROWTH HORMONE ON MUSCLE GLYCOGEN IN ADRENALECTOMIZED AND HYPOPHYSECTOMIZED-ADRENALECTOMIZED RATS[1]

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NORMAL levels of muscle glycogen can not be maintained in the hypophysectomized animal during a fast. The administration of pituitary growth hormone lessens the glycogen depletion, and this hormonal activity is spoken of as the glycostatic effect (1, 2). Illingworth and Russell (3) investigated the glycogen response to purified growth hormone in animals with pituitaries intact. They found that in the normal animal glycogen levels were elevated significantly six hours after injection, whereas the hormone was relatively ineffective in acutely treated adrenalectomized rats. Recently, however, even in the normal animal muscle glycogen has been reported to be unaffected by growth hormone in short term experiments (4, 5). The object of the present experiments was to investigate the effects of purified growth hormone on muscle glycogen as affected by the pituitary and adrenal glands, as well as by the duration of hormone treatment.

MATERIALS AND METHODS

Animals employed were male and female rats of the Long-Evans strain, 3 to 4 months of age. The weight range for the females was 170 to 230 grams and for the males 200 to 300 grams. The animals were fed a diet of calf meal pellets and water, *ad libitum*. Hypophysectomized animals were offered 10% glucose, and adrenalectomized animals 1% NaCl in addition to regular drinking water. Adrenalectomized-hypophysectomized rats received both the salt and glucose solutions, and adrenodemedullated rats were offered salt water for a period of two to three weeks after enucleation.

The operations were carried out under ether anesthesia using standard procedures. The adrenals of the demedullated rats were enucleated 30 days before the animals were used experimentally. Hypophysectomy of the adrenodemedullated animals was performed about a month after demedullation, and the animals were used three days later. The hypophysectomized-adrenalectomized rats were prepared by removing first the adrenals and then the pituitaries 10 to 14 days later. These animals were employed experimentally 3 days after the latter operation. Adrenalectomized animals were used 10 to 14 days after surgery. All operations were checked for completeness at autopsy.

The experiments were divided into three Groups: Group I dealt with normal, adrenal-

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ectomized, and adrenodemedullated female rats. The animals of Group II were either hypophysectomized, hypophysectomized-adrenalectomized, or hypophysectomized-adrenodemedullated females. Male animals were used in Group III and were either normal, adrenalectomized, hypophysectomized, or hypophysectomized-adrenalectomized. Growth hormone was administered prior to autopsy for 3 days to Groups I and II, and for either 6 or 24 hours to the animals of Group III. In all of the experiments saline injected control rats were investigated simultaneously with the growth hormone injected animals.

The growth hormone[2] was weighed out and dissolved in .9% NaCl immediately before use. It was injected intraperitoneally in a volume of .05 to .1 ml. In all experiments in which the hormone was injected for 24 hours or longer it was given twice daily in equally divided doses.

During the 24 hour period previous to autopsy the animals were deprived of food and glucose. The fast extended approximately from 8 A.M. to 8 A.M. At the termination of the fast the animals were anesthetized with Nembutal (6 mg./100 gms. body weight) and, in the order given, the following muscles were removed for glycogen analysis: rectus femoris, a section of the abdominal muscle wall, and one half of the diaphragm. The muscles were weighed immediately, placed in tubes containing 30% KOH, and allowed to digest completely in a boiling water bath. Details of these procedures, and of the method of subsequent glycogen determination are given in a previous report (6). The glycogen values are presented as milligrams of glucose per 100 gm. of muscles, wet weight.

EXPERIMENTAL

Group I: These animals were injected with growth hormone at a dosage level of either 125 or 375 μg. per 100 gms. body weight per day. It can be seen in Table 1 that none of the three muscles from the normal animals injected with the lower dosage showed a response to the hormone, and with the higher amount only the abdominal muscle was affected significantly. In the adrenalectomized animals consequent to injection with the 125 μg. dosage the rectus femoris muscle had a glycogen content significantly above the control value, while with the 375 μg. level the abdominal and diaphragm muscles had higher glycogen stores than did their controls. In the adrenodemedullated animals of this group the hormone was injected only at the dosage of 375 μg./100 grams. It can be seen that in these animals the rectus femoris and abdominal muscles were affected significantly although the diaphragm was not.

Group II: The animals of this group were treated with 125 μg. of growth hormone per 100 gms. body weight per day. As shown in Table 2 the rectus femoris and abdominal muscles from all three operated categories in this group had glycogen values considerably higher than their respective controls. In the diaphragm, however, a significant result was achieved only in the hypophysectomized-adrenodemedullated animals.

Group III: The result of Illingworth and Russell (3) in which purified growth hormone was found not to have a significant glycostatic effect in

[2] The growth hormone (22KR2) was generously supplied by The Armour Laboratories, Chicago, through the courtesy of Mr. I. M. Bunding.

TABLE 1. CHRONIC EFFECT OF GROWTH HORMONE ON MUSCLE GLYCOGEN IN NORMAL, ADRENALECTOMIZED, AND ADRENODEMEDULLATED FEMALE RATS

Treatment†	No. of animals	Rectus femoris	Abdominal muscle	Diaphragm muscle
		Glycogen (mg./100 gm. muscle)		
Normal				
Control	6	522 ± 21	599 ± 20	303 ± 39
Growth hormone (125 μg.)‡	6	541 ± 8	611 ± 10	338 ± 12
Control	5	467 ± 13	582 ± 16	301 ± 14
Growth hormone (375 μg.)	5	526 ± 30	695 ± 37*	294 ± 14
Adrenalectomized				
Control	6	399 ± 10	507 ± 13	371 ± 15
Growth hormone (125 μg.)	6	490 ± 14**	539 ± 12	354 ± 18
Control	7	357 ± 12	433 ± 10	322 ± 13
Growth hormone (375 μg.)	7	396 ± 12	516 ± 22*	406 ± 15*
Adrenodemedullated				
Control	7	500 ± 15	646 ± 15	376 ± 19
Growth hormone (375 μg.)	7	560 ± 7*	721 ± 5**	404 ± 11

† All values are from 24-hour starved animals.
‡ Growth hormone dosages are for 100 gms. body wt. per day for 3 days.
* $P < .05$ that the difference between this value and respective control is due to chance.
** $P < .01$ that the difference between this value and respective control is due to chance.

TABLE 2. CHRONIC EFFECT OF GROWTH HORMONE ON MUSCLE GLYCOGEN IN HYPOPHYSECTOMIZED, HYPOPHYSECTOMIZED-ADRENALECTOMIZED, AND HYPOPHYSECTOMIZED-ADRENODEMEDULLATED FEMALE RATS

Treatment†	No. of animals	Rectus femoris	Abdominal muscle	Diaphragm muscle
		Glycogen (mg./100 gm. muscle)		
Hypophysectomized				
Control	6	261 ± 4	328 ± 14	249 ± 28
Growth hormone‖	12	430 ± 13**	450 ± 18**	224 ± 9
Hypox.-adrenalectomized				
Control	5	337 ± 7	330 ± 9	351 ± 37
Growth hormone‖	7	439 ± 14**	433 ± 25*	424 ± 27
Hypox.-adrenodemedullated				
Control	6	357 ± 27	417 ± 23	347 ± 19
Growth hormone‖	13	505 ± 17**	594 ± 10**	417 ± 14*

† All values are from 24 hour starved animals.
‖ 125 μg. growth hormone per 100 gms. body wt. per day for 3 days.
* $P < .05$ that the difference between this value and respective control is due to chance.
** $P < .01$ that the difference between this value and respective control is due to chance.

the absence of the adrenals[3] was obtained from acute experiments in which adrenalectomized male rats were injected with the hormone only 6 hours before autopsy. To repeat these short term conditions the following experiments were conducted. Normal, adrenalectomized, hypophysectomized, and hypophysectomized-adrenalectomized male rats were given a single

[3] Due to a misprint in the paper by Illingworth and Russell (3) P values for gastrocnemious and diaphragm muscle glycogen read, "<.05" for differences between control and treated adrenalectomized groups. This should have read, ".1–.05."

injection of 500 μg. of growth hormone per 100 gms., 18 hours after the beginning of the fast, i.e., 6 hours before autopsy. In the normal animals the rectus femoris and diaphragm, but not the abdominal muscles responded significantly, while in the hypophysectomized rats the response to the hormone was not significant in any of the muscles examined. The abdominal muscle from the injected adrenalectomized rats exhibited a significantly higher glycogen content than did their controls, but the other two muscles were not affected. The hypophysectomized-adrenalectomized animals showed significant responses in the abdominal and diaphragm muscles but not in the rectus femoris.

An additional experiment was carried out to duplicate conditions of Bennett and Perkins (7) under which they found crude pituitary extracts glycostatically effective in the hypophysectomized-adrenalectomized animal. These workers injected their animals at the beginning and during the 24 hour fasting period. In the present experiments hypophysectomized-adrenalectomized rats were injected with 500 μg. of growth hormone per 100 gms. weight. The dosage was divided, with half being given at the beginning of the fast and the other half 12 hours before autopsy. Table 3 shows that the rectus femoris of the injected animals had a significantly higher level of glycogen than did this muscle in the controls. The abdominal muscle glycogen from the injected group, although averaging much higher

TABLE 3. ACUTE EFFECT OF GROWTH HORMONE ON MUSCLE GLYCOGEN AS INFLUENCED BY THE PITUITARY AND ADRENAL GLANDS IN MALE RATS

Treatment†	No. of animals	Rectus femoris	Abdominal muscle	Diaphragm muscle
		Glycogen (mg./100 gm. muscle)		
Hypophysectomized-adrenalectomized				
Control	5	369 ± 9	354 ± 21	336 ± 41
Growth hormone‖	7	492 ± 22**	458 ± 35	366 ± 42
Control	6	310 ± 9	389 ± 16	358 ± 28
Growth hormone‖	7	366 ± 7**	419 ± 18	426 ± 37
Control	6	342 ± 18	352 ± 9	290 ± 14
Growth hormone‡	7	363 ± 13	392 ± 13*	345 ± 12*
Hypophysectomized				
Control	7	225 ± 12	293 ± 8	155 ± 10
Growth hormone‡	7	236 ± 14	288 ± 14	191 ± 20
Adrenalectomized				
Control	7	407 ± 9	445 ± 16	342 ± 14
Growth hormone‡	7	432 ± 8	495 ± 8*	381 ± 12
Normal				
Control	7	443 ± 13	525 ± 29	314 ± 17
Growth hormone‡	7	478 ± 5*	575 ± 12	374 ± 17*

† All values are from 24 hour starved animals.
‖ 500 μg. growth hormone per 100 gms. body wt. in divided doses during 24 hour fast.
‡ 500 μg. growth hormone per 100 gms. body wt. 6 hours before autopsy.
* $P < .05$ that the difference between this value and respective control is due to chance.
** $P < .01$ that the difference between this value and respective control is due to chance.

than the controls was not statistically altered. The diaphragm glycogen, likewise, was not significantly affected by the hormone. This last experiment was repeated six months later, and although the absolute values show some variation, the results were essentially the same, as can be seen in Table 3.

DISCUSSION

It is clear that under certain conditions purified pituitary growth hormone can exert its myoglycostatic effect in the absence of the adrenal glands. One of the factors which modified this effect was the length of time that the hormone was injected prior to muscle glycogen analysis. When injected for 3 days there was no distinction between the effects of growth hormone in hypophysectomized and hypophysectomized-adrenalectomized rats, and likewise in the animals with pituitary intact the hormone was as effective in the adrenalectomized as in the normal and adrenodemedullated rats.

In the hypophysectomized-adrenalectomized rats injected only during the 24 hour fasting period there was some indication that the absence of the adrenals may have affected the glycostatic response. It has previously been reported (3, 6, 7) that under these circumstances growth hormone is effective in all of the several muscles examined in the hypophysectomized rat. In the present experiments using the doubly operated animals, only one muscle, the rectus femoris, showed a value significantly different from the controls. The work of Bennett and Perkins (7) using pituitary extracts rich in growth hormone, injected during the 24 hour fast, indicated that there was no difference between the myoglycostatic response of the hypophysectomized and hypophysectomized-adrenalectomized rat. These workers, however, apparently employed only the gastrocnemious muscle. As regards glycogen maintenance under the above conditions, the leg muscles, thus, would seem to be less dependent than some of the other muscles upon the adrenal secretions.

In the animals injected only 6 hours before autopsy the presence or absence of the adrenals did not appear to be the deciding factor as to whether or not growth hormone maintained muscle glycogen. Statistically significant differences were obtained somewhat erratically, and the literature, too, was found to be inconsistent in this area. Russell and Bloom (4) reported that in the normal intact rat injected with growth hormone six hours before autopsy, glycogen levels of the gastrocnemious and diaphragm muscles were not significantly altered from the control values. However, under reportedly the same conditions, Illingworth and Russell (3) a few years earlier had demonstrated a significant glycostatic effect of growth hormone in these same two muscles. The results of the experiments reported herein are essentially in agreement with those of these latter workers. A slight, but significant, effect was also found in one muscle of the adrenalectomized animals.

In all of the acute experiments, even when differences between control and injected groups were statistically significant they were not great. Perhaps these varying results can be resolved if the action of growth hormone is presumed to be predominantly glycostatic, and to have little effect in causing an increase in glycogen accumulation. When the hormone is injected 18 hours after the fast has begun, i.e., only 6 hours before the muscles are analyzed, glycogen already has reached a fairly low level. The level may be so low that differences between control and injected groups are difficult to detect, even though 6 hours may be sufficient time for the hormone to stabilize the glycogen. Food intake previous to fasting is a factor which affects the ultimate muscle glycogen levels (8). Thus, it would seem that pair feeding prior to the fast may be one approach in elucidating the short term glycostatic ability of growth hormone in either the presence or absence of the adrenals.

It is obvious that muscle glycogen, *per se*, can not be categorized in respect to its response to growth hormone. Specific muscle, endocrine state, and duration of treatment are among the variables that must be stipulated. It is also possible that the sex of the animal may be an important consideration. Since females were used in Groups I and II, and males in Group III, a reliable comparison between these data may not be possible.

SUMMARY

Several muscles of fasting normal, adrenalectomized, and adrenodemedullated rats were shown to respond to the glycostatic action of purified growth hormone when it was given in sufficient dosage over a three day period. With this same injection period, growth hormone also was glycostatically effective in the hypophysectomized, hypophysectomized-adrenalectomized, and hypophysectomized-adrenodemedullated rat. In the hypophysectomized-adrenalectomized animals injected with growth hormone only during the 24 hour fast, just one of the three muscles analyzed retained glycogen levels significantly above the uninjected controls. When growth hormone was injected 6 hours previous to the termination of the fasting period the results were somewhat variable. The factors controlling the glycostatic effectiveness of growth hormone under these acute conditions remain to be resolved.

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THE ROLE OF THE LIVER IN THE INACTIVATION OF THE MELANOCYTE STIMULATING HORMONE IN THE BODY

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WHEN melanocyte stimulating hormone (MSH) is injected into an animal, the blood level of MSH decreases rapidly thereafter (1). As only a small portion of injected MSH is excreted in the urine (2), the hormone is either rapidly destroyed or bound by tissue in the body.

As the inactivation of posterior pituitary hormones (3, 4) and gonadotrophic hormone (5, 6) by the liver has been reported previously, the role of liver in the rapid disappearance of MSH from the circulation has been studied. Previously Eser and Tuzunkam (7) reported the inactivation of MSH by the liver of rat, pigeon and frog *in vitro* but Durlach and Cachin (8) could not observe the inactivation of MSH by the liver *in vivo*.

In the present study MSH was administered to hepatectomized dogs and to dogs with injured liver and the disappearance rate of injected MSH from the circulation in the dogs was compared with that in normal dogs. In addition, the level of MSH in the portal vein and in the hepatic vein was compared after intravenous injection of MSH into normal dogs. In the second part of the study, inactivation of MSH was tested after incubation with homogenates of liver and other tissues.

MATERIALS AND METHODS

Hog MSH used in this study was a lyophylized powder prepared by the Armour Laboratories, MSH activity was 1.4×10^5 U./mg.[1], with ACTH activity of 1.0 U./mg. and with very little oxytocic and vasopressive activity. This powder was dissolved in physiologic saline for injection. Dog MSH used in this study was prepared in this laboratory in the following way. The posterior pituitary glands from 21 dogs were dehydrated with acetone and then powdered. This powder was then extracted twice with 10 cc. of 0.25% acetic acid. The extract was neutralized with 0.1 N NaOH and the total volume was made to 40 cc. One cc. of this extract had MSH activity of approximately 1×10^5 U. The ACTH activity and oxytocic and vasopressive activity of this extract were not estimated but it is assumed that certain amounts of these and other pituitary hormones were contained in this extract.

Dogs weighing 8 to 10 kg. were used for the experiments. Dogs were anesthetized with 3–4 cc. of 0.6% pentobarbital just before the injection of MSH or just before the

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[1] The unit of MSH referred to is that defined by Shizume, Lerner and Fitzpatrick (1954).

operation of hepatectomy. For the experiment with hog MSH, 28×10^5 U. were injected into an extremity vein and for the experiment with dog MSH, about 10×10^5 U. were injected. The level of MSH in the blood was estimated 5 min., 10 min., 15 min., 30 min., 1 hour, 2 hours, 3 hours, 4 hours and 5 hours after injection. Each time, a blood sample of approximately 3 cc. was withdrawn from the femoral vein and kept in the refrigerator. When all blood specimens were withdrawn, serum was separated by centrifugation and the MSH content in the serum was assayed by the method reported by Shizume, Lerner and Fitzpatrick (9). Two pieces of frog skin were used for each assay. When the concentration of MSH in the serum was high, the serum was diluted to the proper degree and used for the bioassay.

Hepatectomy was performed by the method of Nishimura and Hori (10). The dog was fixed in a supine position, and the peritoneal cavity was opened under pentobarbital anesthesia. After ligating the hepatic artery and common bile duct, the liver was separated from its surroundings by cutting the hepatic ligaments. To facilitate clamping

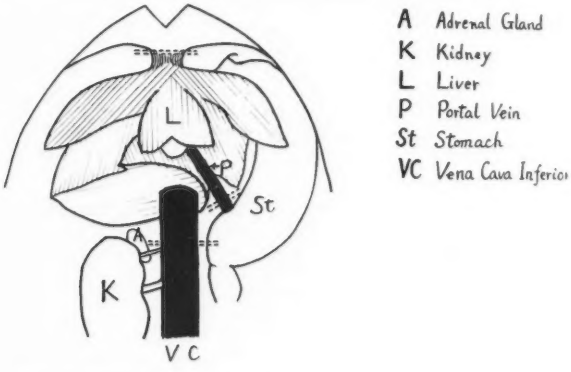

FIG. 1. Sites of clamping shown with double dotted lines.

that portion of the inferior vena cava behind the liver was dissected from the posterior abdominal wall. Then using rubber-shod clamps, the portal vein was obliterated at a point 3 cm. from the liver, the inferior vena cava was clamped just proximal to the branching of the hepatic vein superiorly and just proximal to the branching of the suprarenal vein inferiorly as shown by the double dotted lines in Figure 1. The liver was extirpated and the glass cannula shown in Figure 2 was filled with saline and was connected with the portal vein and the upper and lower parts of the inferior vena cava, and the clamps were removed. As shown in Figure 2, blood in the portal vein then flowed into the inferior vena cava directly. Several cannulas with different lengths between 4.5 cm. and 6.0 cm. were prepared and the one with the proper length was used for each experiment. As dogs die of hypoglycemia soon after hepatectomy, 15% glucose solution was given intravenously at the rate of 0.5 cc./min. By this procedure the dogs survived more than 12 hours after operation. MSH was injected after the peritoneum was closed. Control dogs did not receive laparotomy.

To produce liver injury in the dog, carbon tetrachloride was given by stomach tube in a dose of 0.5 cc./kg. a day for 4 or 5 days. On the day following the last dose of carbon tetrachloride MSH was injected into an extremity vein and the blood levels were studied. After each experiment, a portion of liver was taken and examined histologically. Necrosis of the hepatic cells around the central vein and diffuse infiltration of fat was observed in all 4 dogs used in the experiment.

When blood samples were to be taken from the portal and hepatic veins, laparotomy was performed just before the injection of MSH and blood was taken from the portal vein and from the hepatic vein directly and at approximately the same times. When blood was withdrawn from the hepatic vein, the needle was inserted into the hepatic vein at the edge of the liver, pointed in the direction of the liver, and blood was withdrawn slowly in order to avoid the inflow of blood from the inferior vena cava. Bile was taken by inserting a polyethylene tube into the common bile duct under laparotomy.

Homogenates of dog tissues were prepared from exsanguinated animals. To 300–500 mg. of tissue, 10 volumes of cold physiologic saline solution were added and homogenization was accomplished in the Potter-Elvehjen glass homogenizer.

One hundred units of MSH were mixed with 1 cc. of the homogenate and incubated for 60 minutes at 37° C and the MSH content of the homogenate was estimated by the method of Shizume, Lerner and Fitzpatrick (9).

In some experiments the homogenates were boiled for 5 minutes before incubation and in others incubation was performed at 4° C.

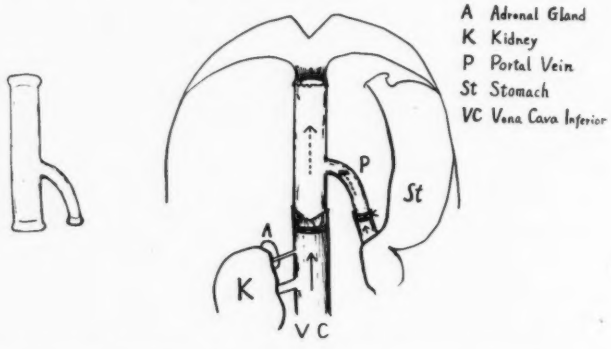

FIG. 2. Glass cannula used for hepatectomy (left). Showing the cannula connecting the portal vein and inferior vena cava (right).

RESULTS

1. Blood levels of injected hog MSH in normal dogs and in hepatectomized dogs.

The blood levels of MSH after the injection of hog MSH in 3 normal dogs and in 5 hepatectomized dogs are shown in Figure 3. The disappearance rate of MSH in the hepatectomized dogs was definitely retarded as compared with that in normal dogs.

2. Blood levels of injected dog MSH in normal dogs and in hepatectomized dogs.

As hog MSH is a foreign substance for the dog, dog MSH was injected into 2 normal dogs and into 2 hepatectomized dogs. As shown in Figure 4, the level of MSH in the blood decreased rapidly in normal dogs and retardation of the disappearance rate was observed in hepatectomized dogs.

Dog MSH is difficult to obtain in sufficient quantity and is more contaminated with other pituitary hormones than hog MSH prepared by the Armour Laboratories. Since similar results were obtained with hog MSH

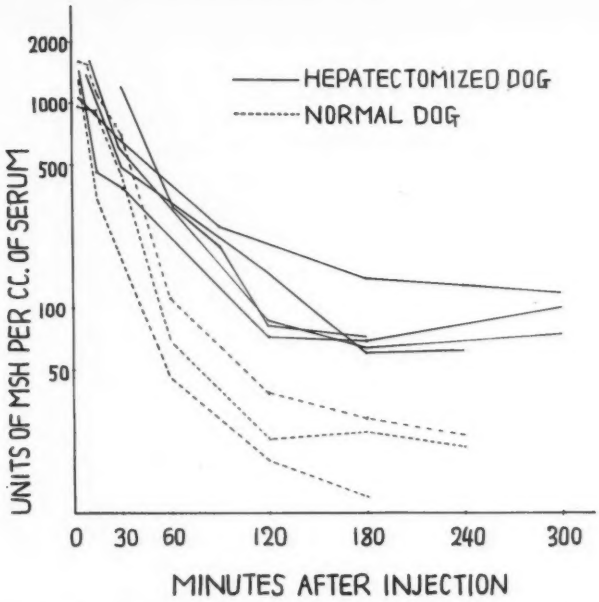

FIG. 3. Blood levels of MSH after intravenous injection of 28×10^5 U. of hog MSH into 3 normal dogs and 5 hepatectomized dogs. The ordinate is in log scale.

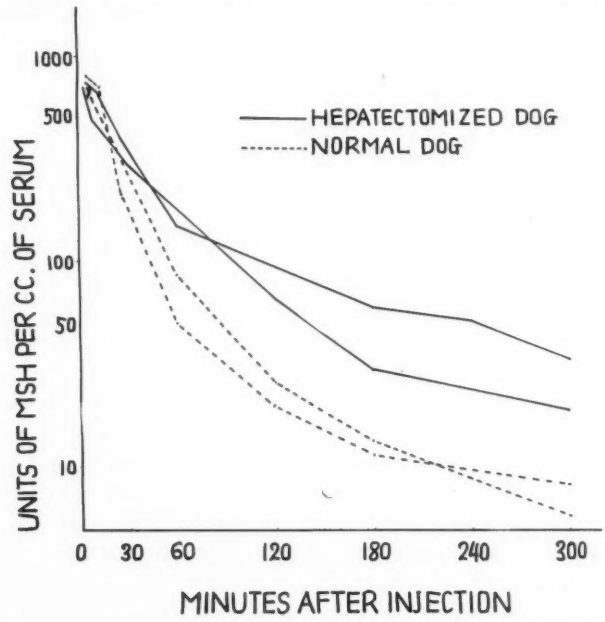

FIG. 4. Blood levels of MSH after intravenous injection of 10×10^5 U. of dog MSH into 2 normal dogs and 2 hepatectomized dogs. The ordinate is in log scale.

as with dog MSH in the above experiment, in the following experiments, hog MSH was used.

3. Blood levels of injected MSH in dogs with liver injury.

Figure 5 shows the blood level of MSH after intravenous injection of hog MSH in 4 dogs with liver injury by carbon tetrachloride. The disappearance rate of MSH in dogs with liver injury was retarded as compared with that in normal dogs. Compared with the disappearance rate in hepatectomized dogs shown in Figure 3, the retardation seemed to be less marked.

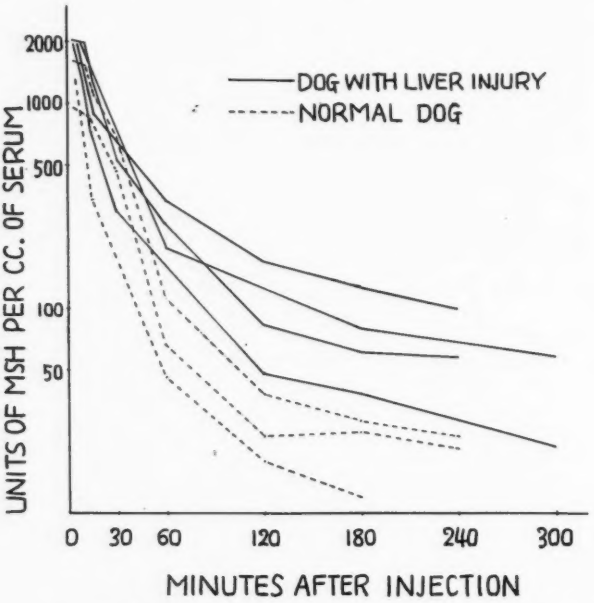

FIG. 5. Blood levels of MSH after intravenous injection of 28×10^5 U. of hog MSH into 4 dogs with liver injury by carbon tetrachloride compared with that in normal dogs. The ordinate is in log scale.

4. Blood levels of injected MSH in the portal vein, hepatic vein, and in the bile.

The levels of MSH in the portal vein and hepatic vein 5 min., 30 min., and 60 min. after intravenous injection of hog MSH into 3 normal dogs are shown in Table 1. The level of MSH in the portal vein was always higher than that in the hepatic vein.

During this experiment, a polyethylene tube was inserted into the hepatic duct, and bile was collected for 60 minutes after the injection of MSH. No appreciable amount of MSH was detected in the bile in any of 3 dogs.

5. Inactivation of MSH after incubation with homogenates of various tissues under different conditions.

TABLE 1. MSH IN THE PORTAL VEIN AND IN THE HEPATIC VEIN AFTER INTRAVENOUS INJECTION OF 28×10^5 U. OF MSH

(Figures show units of MSH per cc. of serum)

Dog No.	No. 1			No. 2			No. 3		
Time after injection	5 min.	30 min.	60 min.	5 min.	30 min.	60 min.	5 min.	30 min.	60 min.
Blood from portal vein	1600	980	560	1000	370	300	1240	960	440
Blood from hepatic vein	1100	820	260	820	300	164	620	520	230

The percentage of MSH inactivated after incubation with homogenates of various tissues from dogs for 60 minutes at 37° C is shown in Table 2. It was found that liver, kidney and muscle have systems capable of inactivating MSH during *in vitro* incubation and the greatest activity was found in homogenates of liver tissue. Appreciable inactivation was not observed in homogenates of spleen, brain and serum under these conditions. When homogenates of liver, spleen and muscle were boiled before incubation, or incubation with these tissues was performed at 4° C instead of 37° C, no inactivation was observed.

DISCUSSION

The experiments reported here show that the disappearance of injected hog MSH from the circulation was definitely retarded in the hepatectomized dogs. It was also shown that after injection of hog MSH the level of MSH in the portal vein was always higher than that in the hepatic vein and MSH was not excreted in the bile. These results indicate that MSH is either inactivated or bound by the liver. As hog MSH is a heterogenous substance for the dog, it is conceivable that hog MSH is trapped by the reticuloendothelial system in the liver. However, when dog MSH was injected into normal dogs, it disappeared rapidly from the circulation and the disappearance rate was retarded in hepatectomized dogs. In addition it was also shown that the rate of disappearance of MSH from the circulation

TABLE 2. PERCENTAGE OF HOG MSH INACTIVATED BY INCUBATION WITH HOMOGENATES OF TISSUES FROM DOGS

No. of experiment	Liver	Kidney	Muscle	Spleen	Brain	Serum
1	72					
2	90	60				<10
3	90	66		<10		<10
4	74	52	52	<10	<10	<10
5	65		32	<10	<10	<10
6	58	42	24	<10		<10
7	80	69	56	<10		
Average	76	58	41	<10	<10	<10

was definitely retarded by carbon tetrachloride induced liver injury. This is predominantly an injury to the hepatic cells rather than the reticuloendothelial system. These results therefore suggest that the reticuloendothelial system in the liver is not greatly concerned with the rapid disappearance of MSH from the circulation.

The experiments in the second part of the study showed that homogenates of liver, kidney and muscle contain an enzyme like system capable of inactivating MSH. The deduction of its enzymatic nature is based upon the thermolability and disappearance of reaction at 4° C. The specificity of the system has not been studied. As MSH is a polypeptide (11), it is possible that the enzyme is a polypeptidase.

Although the inactivation of MSH by homogenates of spleen, brain and serum has not been observed, the presence of such an inactivating system in these tissues cannot be excluded since the study reported here did not detect inactivation which was less than 10 per cent. The present study shows that liver is one of the main sites of inactivttion of injected MSH. Whether this inactivating system plays a physiological role in the destruction of endogenous MSH has still to be determined. Elevated values of blood MSH observed in some patients with liver cirrhosis in this laboratory suggest that the system in the liver may play such a role.

SUMMARY

When hog or dog MSH was injected into dogs, the blood level of injected MSH decreased rapidly thereafter. When hog or dog MSH was injected into hepatectomized dogs, the rate of disappearance was definitely retarded as compared with that in normal dogs.

Similar retardation, although in lesser degree, of the disappearance of injected MSH from the circulation, was observed in the dogs whose livers were injured with carbon tetrachloride.

When MSH was injected into normal dogs, the level of MSH in the portal vein was always higher than that in the hepatic vein, and MSH was not found in the bile.

When MSH was incubated with homogenates of various tissues at 37° C, homogenate of dog liver inactivated MSH more efficiently than did homogenates of kidney and muscle. When these homogenates were boiled before incubation or incubation was performed at 4° C, no inactivation was observed.

These results indicate that liver is one of the main sites of inactivation of MSH and the inactivation in the liver is performed by some enzymatic system.

Acknowledgments

We want to thank Prof. Shigeo Okinaka for interest in and help for these studies, Dr. A. B. Lerner for help given throughout these studies, Dr. Irby Bunding of the

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CORTISONE EFFECT ON OVULATION IN THE FROG[1]

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IN A recent report on estrogen-induced adrenal hyperplasia we referred to the possibility that adrenal hormones might play a supporting role in the induction of ovulation (1). The suggestion derived from the observation that high levels of estrogens, such as those which ordinarily precede ovulation, affect not only the gonadotrophic output of the hypophysis but also the release of ACTH. Since the mechanics of hormonal induction of ovulation are far from completely understood, it may tentatively be assumed that the following chain of reactions assists the gonadotrophic complex in eliciting the rupture of ovarian follicles: *high estrogen*→(*hypophysis*) *ACTH*→(*adrenal cortex*) *cortisone*→(*ovary*) *ovulation.*

Considering the difficulties of proper timing and selection of adequate dosages in experimental testing, cortisone should have a better chance of producing positive results than either ACTH or estradiol, the more distant inductors in the reaction sequence. The present experiments were initiated to test these assumptions.

MATERIALS AND METHODS

During the winter months, jumbo females of *Rana pipiens* hold in their ovaries about two thousand full grown follicles with mature eggs ready for ovulation. In preparation for the experiments the frogs were transferred from storage at 3° C to a laboratory with a controlled temperature of 20° C. Under the conditions described here spontaneous ovulation was never seen to occur before March. In December the implantation of three donor female hypophyses into the dorsal lymph sac usually causes ovulation in 80 to 100% of the recipient females within 48 hours. In January and February two hypophyses produce the same effect. Hypophyses are always transplanted intact, avoiding irregular distribution and possible losses of material. In preliminary experiments it was ascertained that ovulation is not elicited by injections of any one of the three hormones alone: estradiol, ACTH or cortisone. To bring the females to ovulation threshold they were implanted with two hypophyses in December, or with one in January or February. Injections of estradiol, ACTH, and cortisone acetate were administered at the same time. In a third set of experiments additional estradiol was injected 24 and 12 hours before the implantation of the hypophysis. After 24, 48 and 72 hours the success of the treatments was checked by gently compressing the abdomen, forcing ovulated eggs out through the cloaca. At the end of the observation period the animals usually were killed and the ovaries and uteri examined directly. One group of experiments was carried out with the ovaries *in vitro*. The technique employed in this series will be described together with the

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results. Two hundred forty-one female frogs served as test animals. About 470 more females of similar size (body lengths 9 to 10 cm.) were used for preliminary assays and as hypophyseal donors.

EXPERIMENTS

1. The results of a first group of tests are summarized in Table 1. The "sub-threshold" hypophyseal implants led to ovulation in 29% of the otherwise untreated females (controls). Parenteral injection of 5 mg. cortisone acetate in aqueous suspension, simultaneous with hypophysis implantation, resulted in a very pronounced and statistically highly signifi-

TABLE 1. OVULATION RESPONSE WITHIN 48 HOURS TO IMPLANTED HYPOPHYSES, SUPPLEMENTED WITH HORMONE INJECTIONS

Supplement	No. frogs	No. ovulated	χ^2*	P*
Cortisone (5 mg.)	40	28 (70%)	12.7	<0.001
ACTH acetate (4 I.U.)	23	14 (61%)	5.2	<0.03
Estradiol (2 mg.)	30	8 (27%)	0.003	>0.90
Control	45	13 (29%)	—	—

* Calculations were made versus unsupplemented group (control), with corrections for continuity.

cant *increase in ovulation percentage* (70%). Similar injections of ACTH doubled the control percentage, while estradiol at the dose of 2 mg. per animal was ineffective.

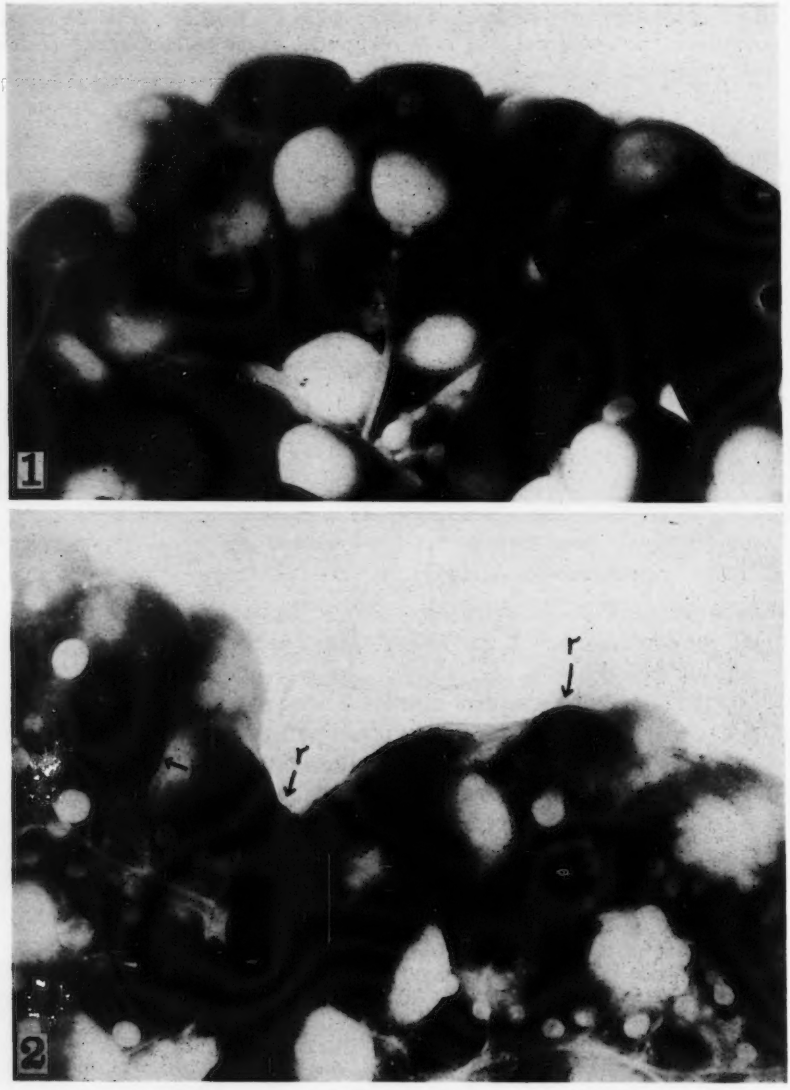
2. In the course of these experiments it was noticed that cortisone injection not only increased the number of animals ovulating, but that it also *accelerated the ovulation process.* This is illustrated by the results of the second experimental group (Table 2), in which ovulations of the first day are recorded separately. Even at the low dose of 1 mg. cortisone per animal more than half of the females ovulated during the first 24 hours, i.e., before any of the controls. An increase in the cortisone dosage to 25 mg. further enhanced the response, though probably about 5 mg. is adequate for the maximal effect.

3. The evident failure of estradiol to stimulate ovulation under the conditions of the first experimental group (Table 1) may be due to any one of several circumstances. The form of administration differed from that of cortisone and ACTH. Estradiol was dissolved in sesame oil and was given into the dorsal lymph sac. In a third experimental group the crystalline

TABLE 2. ACCELERATION OF INDUCED OVULATION BY CORTISONE

Supplement*	After 24 hours	After 48 hours	No. frogs
Cortisone (1 mg.)	11 (55%)	13 (65%)	20
Cortisone (25 mg.)	15 (79%)	17 (89%)	19
Control	0	5 (50%)	10

* Each frog was implanted with one hypophysis.



FIGS. 1–3. Isolated ovarian lobes of a female *Rana pipiens*, 24 hours after having been placed in 20 ml. of amphibian Ringer's solution. **1** Control. **2** With the addition of 1 mg. of cortisone acetate: *r* ruptured follicles. **3** With the addition of 1 mashed hypophysis and 1 mg. of cortisone acetate: at the top, two ovulated eggs; *e* empty follicles; *o* ovulating follicles; all ×10.

hormone was suspended in 50% propylene glycol and injected into the body cavity. Moreover, the total dosage per animal was changed, and the steroid was administered in three fractions 24 and 12 hours before and at

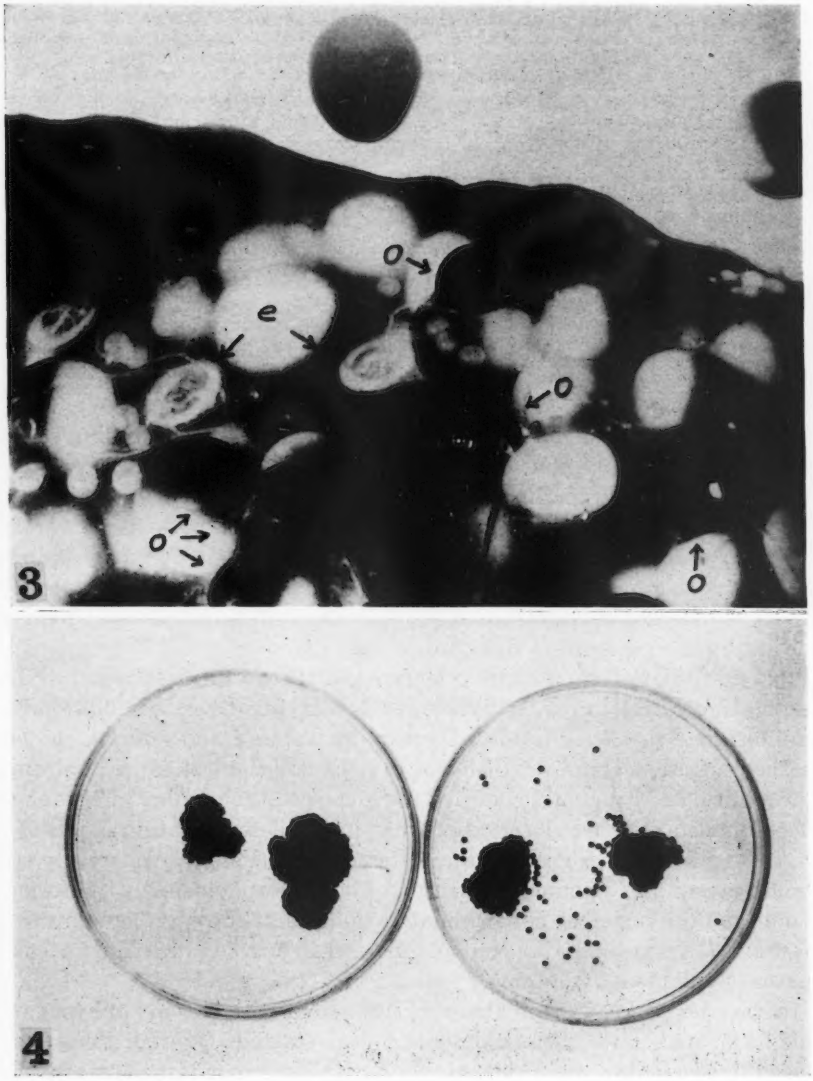


FIG. 4. Isolated ovarian lobes from a female that had received 2 hypophyses implanted into the dorsal lymph sac, 15 hours before killing. Left dish with Ringer's solution only. Right dish with addition of 1 mg. of cortisone acetate. Effect after 24 hours; ×0.55.

the time of hypophyseal implantation. This is itemized in Table 3. The results are still not too clear. However, it is recognized that 2 mg. and 5 mg. per female are very high dosages. The slight increase in ovulation obtained with the smallest dose is suggestive. Possibly even better results might be

TABLE 3. OVULATION ONE TO THREE DAYS AFTER IMPLANTATION OF ONE HYPOPHYSIS SUPPLEMENTED WITH PRECEDING AND SIMULTANEOUS INJECTIONS OF ESTRADIOL. THE CONTROL ANIMALS RECEIVED EACH ONE HYPOPHYSIS AND GLYCOL INJECTIONS IN THE SAME AMOUNT AS THE EXPERIMENTALS

Estradiol injections		No. frogs	Ovulated (hrs. after impl.)			Total ovulated
Dosage	Time*		(24)	(48)	(72)	
5 mg. 5 mg. 10 mg.	24 h. 12 h. 0 h.	13	1	0	2	(15%)
0.25 mg. 0.25 mg. 0.50 mg.	24 h. 12 h. 0 h.	15	2	3	5	(33%)
Control		15	1	2	3	(20%)

* Hours before hypophyseal implantation.

obtained with still smaller dosages. The advance of the breeding season prevents the continuations of tests in the present year.

4. A final set of experiments was designed to test the assumption of a *direct action of cortisone*. Entire ovarian lobes were ligated with silk thread, resected, and placed in Petri dishes with 20 ml. buffered amphibian Ringer's solution, each dish receiving one lobe from the left and one from the right ovary. The literature about the *in vitro* technic may be found by consulting the paper of Wright and Hisaw (2).

In a *first group* the lobes were taken from four females directly out of cold storage and placed in four individual dishes. Hypophyseal stimulation was administered *in vitro*. As may be seen in Table 4 the addition of one mashed pituitary gland per dish was in itself sufficient to cause ovulation. The addition of 1 mg. cortisone acetate increased the number of eggs shed. More important is the observation that incomplete ovulation is initiated by the same dose of cortisone administered alone (Fig. 2). Apparently cortisone causes the opening (rupture) of the morphologically preformed ovulation pore but does not stimulate expulsion of the egg (compare with Figure 1, the non-ovulating control lobe and Figure 3, which illustrates all phases of complete ovulation).

In a *second group*, five females were implanted with two hypophyses and killed 15 hours later, before ovulation had started. Ovarian lobes were placed in two dishes, one being supplied with 1 mg. cortisone acetate. The results were the same in all five cases (Table 4, lower line). Ovulation oc-

TABLE 4. OVULATION FROM OVARIAL LOBES *in vitro*

Hypophysis administered	Number of tests	Control	Cortisone acetate	Hypophysis	Hypophysis and cortisone acetate
In vitro	4	0	(4)*	4**	4***
In vivo	5	—	—	0	5

* Incomplete ovulation (see text).
** Fewer ovulations than in ***.

curred from all of the cortisone-treated lobes, but not from the untreated ones (Fig. 4).

In all the positive cases of both groups ovulation started 15 to 20 hours after the lobes had been placed in the respective solutions.

DISCUSSION

The present experiments were planned to investigate the possible supporting effect of cortisone in ovulation. They provide clear proof of such a facilitating role. However, cortisone is effective only if it acts on follicles that have been brought close to the ovulation threshold by other hormonal factors, among which the LH—progesterone sequence may be assumed to be the most important. Since *in vitro* experiments show that hypophyseal hormones in large dosage may independently produce ovulation, cortisone does not appear to be an indispensable factor.

The starting point of this investigation was the observation that high estrogen levels cause enlargement of the adrenals (1). The same hormonal condition is usually assumed to induce LH release from the hypophysis, at least in mammals (3). Recent work points to an involvement of hypothalamic centers and suggests possibilities of even more complex hormonal mechanisms in ovulation.

SUMMARY

Cortisone and ACTH have been shown to be capable of playing a supporting role in the hormonal control of ovulation in the frog, *Rana pipiens*.

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RESPONSES OF THE DOMESTIC FOWL TO HYPER- AND HYPOGLYCEMIC AGENTS

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RECENTLY, increasing attention has been paid to the effects of various hyper- and hypoglycemic agents in studies of mammalian carbohydrate metabolism. Both cobaltous chloride (1) and Synthalin A (2) have been used to damage the pancreatic alpha cells in efforts to study the role which glucagon-release might play in the carbohydrate scheme; *l*-tryptophan (3) and certain sulfonylureas (4, 5) have been studied for their blood-sugar-lowering effects.

Little work has been done on responses of the fowl to either anti-insulin or insulin-like substances. Yet responses of mammals to these substances prompt the study of them in animals, such as the fowl, whose normal blood-glucose level is quite high compared with that of mammals, but which are in themselves unresponsive to diabetogenic techniques. Herein are reported experiments with two of these substances, cobaltous chloride and 1-butyl-3-p-tolysulfonylurea (Orinase[1]), on carbohydrate metabolism in the domestic fowl. Some experimental responses of chickens of different ages to exogenous glucagon[2] are also reported.

METHODS

These studies were carried out on 186 chickens. All were Single-Comb White Leghorns except those used in one of the Orinase series, which were New Hampshires. All birds were either 6–7 weeks or 12–13 weeks of age as indicated in the text. The birds were maintained on a standard complete chicken mash until the time of experimentation.

Blood samples amounting to either 0.1 or 1 cc. were drawn by cardiac puncture. Blood sugar was determined by Nelson's modification of the Somogyi method (6) and nonprotein nitrogen by the standard micro-Kjeldahl technique. Salts of cobalt, nickel, and iron were injected intravenously into the alar vein as 1% solutions in 0.9% NaCl; glucagon and Orinase were administered (in the same medium) intramuscularly into the shoulder girdle.

During observation periods all birds were immobilized by tying their legs, and they had access to neither food nor water. No anesthetic was used at any time.

RESULTS

Series I. Effect of Cobalt, Nickel and Iron Salts on Blood Sugar Levels. This study was designed to evaluate the chicken's sensitivity to the in-

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[1] Kindly supplied by C. J. O'Donovan, The Upjohn Company, Kalamazoo, Michigan. Lot no. Res. 11,584–2.

[2] Kindly supplied by W. L. Kirtley of the Eli Lilly Co., Indianapolis, Indiana. Lot no. CT-800.

duction of cobalt hyperglycemia and also to determine whether the chemically similar nickelous or ferrous ions would produce effects parallel to those of cobalt. Forty-two 6- to 7-week-old female birds were fasted 12 hours and injected as follows (10 mg./kg. body weight): 11 with cobaltous chloride; 13 with nickelous chloride; 8 with ferrous sulfate. The average amount of any of the metals was 7.6 mg./bird. Ten control birds were injected with 2 cc. of physiological saline.

The results are presented in Figure 1. Only cobalt produced a hyperglycemic response. The initial phase of the blood-glucose rise was similar

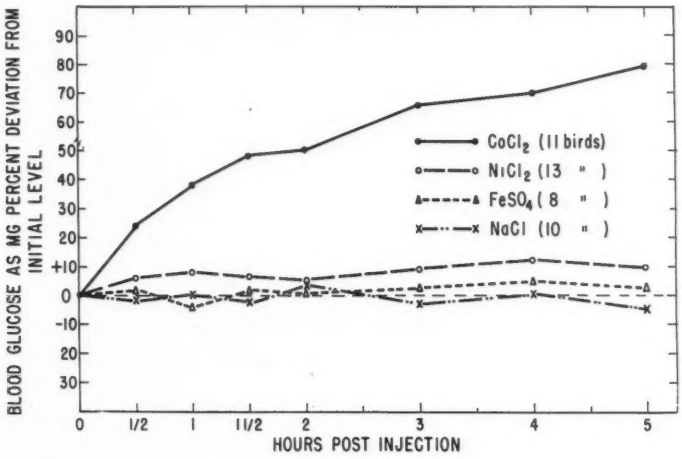

FIG. 1. Effects of injection of cobalt, nickel, and iron salts on the blood glucose of fasting chickens. Each metal was injected at the dose of 10 mg./kg. body weight. The range of initial blood sugar was 188–196 mg. %.

to established effects of glucagon therapy in mammals. However, in contrast to the mammalian response to either glucagon or cobalt, the blood sugar continued to rise for several hours and was still elevated 70–80 mg.% above the fasting pre-injection level at the end of a 5-hour observation period.

Series II. Effect of Glucagon Administration in the Chicken.

Table 1 presents data on nine non-fasted female chickens, 12 weeks old, each injected with 0.2 mg. glucagon. Ten birds injected with 2 cc. of 0.9% saline served as controls. Glucagon therapy caused an immediate hyperglycemia and, as in mammals, the normal blood-sugar level was reattained within 60 to 90 minutes after the injection. These results are in disagreement with those of Snedecor, Mathews and MacGrath (7), who observed a prolonged hyperglycemia persisting four hours after injection of glucagon in 1-day-old turkey poults.

To ascertain whether this discrepancy was due to the age difference,

glucagon (0.2 mg. each) was injected into 11 non-fasted birds which were six weeks old. Since Snedecor *et al.* had postulated that the prolonged hypoglycemic response they observed was due to gluconeogenesis, determinations of non-protein nitrogen were included in this series. The results of this treatment are also shown in Table 1 and may be compared with the results with older birds. It is apparent that the increased blood-sugar response to glucagon therapy was maintained for at least five hours, and in this respect the data from the younger birds are similar to those of Snedecor *et al.* The NPN levels decreased steadily from a pre-injection level of 42.6

TABLE 1. EFFECTS OF GLUCAGON ON BLOOD GLUCOSE AND BLOOD NON-PROTEIN NITROGEN OF CHICKENS

Glucagon, 0.2 mg. per bird was injected IM immediately after withdrawing the initial blood sample. Controls received 2 cc. 0.9% NaCl by the same route.

Treatment	No. birds	Post-injection time in minutes							
		0	30	60	90	120	180	240	300
Twelve-week-old chickens									
		Blood glucose in mg. %*							
Glucagon	9	209±2.0	271±2.3	222±3.4	209±3.2	204±2.2	210±2.8	215±2.1	218±1.9
Control	10	221±2.8	221±2.7	229±3.5	231±4.5	232±1.8	222±4.2	226±2.3	232±1.8
Six-week-old chickens									
		Blood glucose in mg. %*							
Glucagon	11	224±3.4	347±8.2	335±6.5	287±10.7	297±9.8	294±9.9	305±7.6	340±13.4
Control	10	222±3.2	215±3.7	219±4.9	220±3.3	223±3.4	228±3.8	232±2.8	233±2.6
		Blood NPN in mg. %*							
Glucagon	11	42.6±1.5	38.8±1.1	34.7±1.1	32.5±1.4	31.3±1.7	30.6±1.3	25.1±1.0	26.6±1.0
Control	10	41.0±0.3	40.8±1.1	41.7±0.3	41.4±1.1	40.6±0.3	39.2±0.2	39.7±1.2	41.6±1.0

* All values are averages with standard errors of the mean.

mg.% to 26.6 mg.% at the end of five hours (a decrease of 36.6%) indicating a decrease in gluconeogenesis. Glycogen analyses made at the end of the experiment demonstrated at least three times as much liver glycogen in the glucagon-treated birds as in the saline-injected controls (2182 vs. 674 mg.%).

Series III. The Effect of Orinase on the Blood Sugar of Birds.

Since the mechanism of action of Orinase is not completely understood, multiple doses were employed to determine the range of effectiveness in chickens. Sixty-three 12-week-old female chickens were fasted six hours, and Orinase was then administered at dose levels of 10, 25, 50, 200, 400, 1000, and 3000 mg. per kilogram of body weight. All doses were injected intramuscularly except the highest which was pipetted directly into the crop because of the large volume needed.

Table 2 presents the data on the Orinase-treated birds at intervals over a 4-hour period. As little as 10 mg./kg. body weight of this hypoglycemic agent was effective, while the maximum effective dose would appear to have been approximately 200 mg./kg. body weight. The persistent depression of blood glucose at the highest dose level (3000 mg./kg.) may be due

TABLE 2. EFFECT OF ORINASE ON BLOOD GLUCOSE OF 12-WEEK-OLD CHICKENS

All birds were treated, by intramuscular injection except as noted, immediately after withdrawal of the initial blood sample; control birds received 4 cc. of 0.9% NaCl IM.

Orinase dosage, mg./kg.	No. birds	Ave. body wt., gm.	Post-injection time in minutes					
			0	30	60	120	180	240
			Blood glucose in mg. %*					
10	8	1571	200±2.1	154±4.2	163±3.8	171±3.4	172±3.1	194±3.7
25	8	1718	205±2.8	151±6.0	143±5.8	149±8.2	162±8.5	181±6.8
50	8	1571	207±4.9	141±7.4	118±7.3	124±10.1	142±11.2	174±8.6
200	8	1624	198±1.9	116±4.2	79±9.1	75±6.9	80±5.1	97±6.8
400	8	1690	198±2.3	147±8.3	124±13.3	129±12.9	142±14.4	150±13.5
1000	7	1645	193±2.6	119±7.3	95±7.9	108±10.9	110±11.5	120±8.2
3000†	5	1661	190±3.6	128±3.1	99±3.9	71±5.7	60±6.9	58±2.2
Control	11	1673	205±2.1	204±2.2	197±1.7	198±1.7	202±3.2	205±2.9

* All values are averages with standard errors of the mean.
† Pipetted directly into the crop.

to the fact that the drug was administered per os; the slower absorption may account for the prolonged hypoglycemic effect.

It is of interest that during the Orinase trials there was no evidence of hypoglycemic convulsions in any of the birds even though several had blood-glucose levels as low as 32–36 mg.% as compared with the normal of 190–200 mg.%.

In order to determine whether an age difference in response to Orinase might be manifested as with glucagon, eleven 6-week-old birds were fasted 6 hours and injected with the smallest dose (10 mg./kg.) previously employed. Twelve controls were injected with 1.0 cc saline. The results are presented in Figure 2, on which data for the 12-week-old birds receiving the same dose (Table 3) are also plotted for comparison. The younger birds showed considerable resistance to the action of the drug.

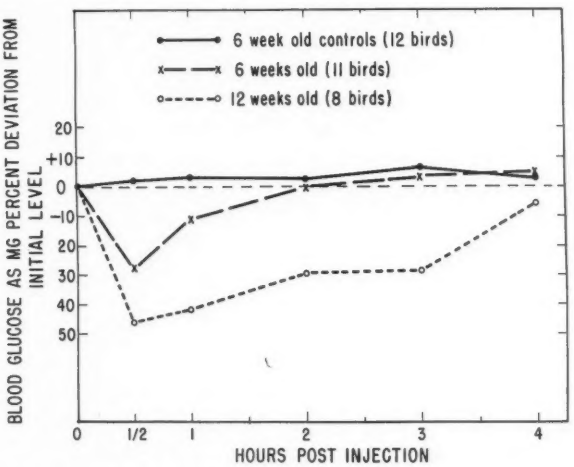

FIG. 2. Effect of age on the response to 10 mg./kg. body weight of Orinase. All birds were fasted six hours prior to use. The initial range of blood glucose was 195–201 mg.%.

Series IV. Response of Orinase-treated Birds to Glucagon Administration. Because of its sensitivity to Orinase, the bird is especially suitable for testing one theory of hypoglycemic mechanism of this substance. If inhibition of glycogenolysis is its major action, an Orinase-treated bird should be resistant to glucagon. Orinase-treated rabbits have been reported to respond to glucagon with increase in blood sugar (8), but the magnitude of the glycemia did not reach that of control rabbits that received similar amounts of glucagon.

Ten non-fasted birds 12 weeks old were pretreated with Orinase, 10

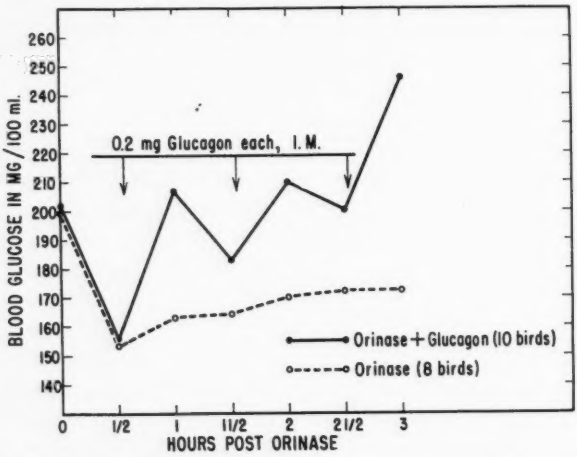

FIG. 3. Effect of repeated glucagon administration to 12-week-old non-fasted birds pretreated with Orinase at the dose level of 10 mg./kg. body weight.

mg./kg. body weight; glucagon was injected (0.2 mg.) 30, 90, and 150 minutes after the Orinase injection. Eight control birds received Orinase only also at 10 mg./kg. body weight. Exogenous glucagon at the dose level used was capable of overcoming the hypoglycemic action of Orinase (Fig. 3). The hyperglycemic agent returned the glucose level of the Orinase-treated birds to normal on the first two injections and created a slight but definite hyperglycemia on the third injection. These observations would suggest that glycogenolysis can occur in birds pretreated with Orinase at a dose level of 10 mg./kg. body weight.

DISCUSSION

The injection of cobaltous chloride into chickens caused a hyperglycemia of a magnitude comparable to that seen in mammals. The hyperglycemia, however, persisted for at least five hours, and at this time there was no indication of a return to the normal avian level. It is unlikely that this marked increase in blood glucose is the result of increased adrenal activity

since neither nickelous chloride, a metallic salt as foreign as cobalt, nor ferrous sulfate could elicit glucogenic responses. A possible direct action of cobalt on the liver, as suggested by Volk, Lazarus and Goldner (1), cannot be ruled out. These investigators observed that injection of cobaltous chloride produced a transitory hyperglycemia in rabbits, but concluded that concurrent histological alterations of the pancreatic islets were independent of the increase in blood glucose.

The lack of histological data in the study presented here prevents any deductions as to the role of pancreatic alpha cells in the cobalt-induced hyperglycemia. However, if cobalt releases glucagon, the response to exogenous administration of glucagon should mimic the response to cobalt. The data presented here indicate that glucagon-induced hyperglycemia is much more marked and more rapidly initiated than is that following cobalt administration. For example, 30 minutes after the injection of glucagon in 6-week-old birds the average blood-sugar level was 112 mg.% above the pre-injection value, whereas in cobalt-treated birds of the same age blood sugar was elevated only 24 mg.% at this interval.

The role of glucagon in the carbohydrate metabolism of the bird is yet to be established. Vuylsteke and de Duve (9) demonstrated the bird pancreas to contain exceptionally large amounts of glucagon (6 times that of mammals), and data presented here and elsewhere (7) show the bird to be very sensitive to this substance. Very likely, then, the amount of glucagon found in the avian pancreas may indicate that the comparatively high normal blood glucose seen in birds is due to a greater glucagon/available insulin ratio. It has been suggested that the persistent hyperglycemia seen in 1-day-old turkey poults following exogenous glucagon therapy may be due to gluconeogenesis (7). This is not consistent with the non-protein nitrogen data presented here (Table 1) in birds injected with glucagon. On the contrary, the steady decrease observed in these values suggests that gluconeogenesis plays little or no part in the response to glucagon.

Only the 12-week-old birds exhibited a response to glucagon similar to that seen in mammals, the blood sugar returning to normal within 90 minutes. The response of younger birds persisted for at least 5 hours, suggesting that the pancreatic islet cells of the latter may be somewhat hypofunctional at this age as compared with the 12-week-old bird.

The inability of the young bird to compensate fully for artificially induced hyperglycemias led to the investigation of the hypoglycemic agent Orinase. Data are available indicating that Orinase acts in either the formation or release of insulin from the mammalian pancreas, as suggested by Goetz, Gilbertsen and Josephson (10) rather than as an anti-insulinase substance (11). The results presented in Table 2 clearly demonstrate that the effective dose for birds is approximately one tenth that previously reported for rats (12). With extremely high doses of Orinase there was no evidence of gross adrenal discharge as has been demonstrated in rats (12); hypo-

glycemia followed all doses. Also, the data presented in Series IV indicate that glycogenolytic pathways are open in birds treated with Orinase, for subsequent glucagon therapy evoked normal or elevated blood-sugar levels.

Of the various theories that have been proposed to explain the hypoglycemic action of Orinase, two of the most promising are (a) that it stimulates insulin formation or release and (b) that it blocks glycogenolytic pathways in the liver. The results presented here with doubly (glucagon and Orinase) treated birds do not support the latter view. On the other hand, the relative resistance of the young bird to Orinase can be explained by islet-cell insufficiency only provided the theory involving Orinase-stimulation of insulin supply is valid. The inability of the young bird to compensate for cobalt- and glucagon-induced hyperglycemia would support this viewpoint.

SUMMARY

Responses of the chicken to hyper- and hypoglycemic agents were studied. Injection of cobaltous chloride caused a moderate hyperglycemia lasting at least five hours; nickel and iron salt injections had no effect on the blood-glucose level.

Exogenous glucagon produced a transitory hyperglycemic response in 12-week-old birds comparable with that reported in mammals, but in 6-week-old birds the blood-sugar elevation lasted for at least five hours. This persistent hyperglycemia does not appear to be due to an effect of glucagon on avian gluconeogenesis since blood non-protein nitrogen declined during the hyperglycemic response.

Dose-response studies in chickens demonstrated that as little as 10 mg./kg. of body weight of injected Orinase was effective in lowering blood sugar; the most effective dose appeared to be 200 mg./kg. body weight. Birds pretreated with Orinase still respond to subsequent glucagon administration, indicating that glycogenolytic pathways are not blocked by the hypoglycemic agent.

Six-week-old birds demonstrated resistance to Orinase administration when compared to 12-week-old birds receiving the same dosage. The inability of the young bird to compensate for artificially induced hyperglycemia as well as its resistance to Orinase administration suggests that the islet beta cells of the 6-week-old bird may be somewhat hypofunctional.

Addendum

Since submission of this paper Dr. I. A. Mirsky (*Endocrinology* **61**: 148. 1957) has been successful in demonstrating that pancreatectomized fowl respond to Orinase administration. These results, in apparent conflict with the hypothesis stated above, suggest the existence of alternate mechanisms. Further experiments are in progress in this laboratory in attempt to elucidate further the mechanisms involved.

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THE BIOASSAY OF PROGESTERONE[1]

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IT WAS first observed by Lutwak-Mann (1) that gonadotrophins, progesterone and certain other luteoids injected into estrous adult or estrogen-primed immature rabbits produced marked increases in the content of uterine carbonic anhydrase, the extent of which depended upon the dosage, and this was accompanied by histological evidence of progestational proliferation (2). It was therefore suggested that the measurement of endometrial carbonic anhydrase might be used as a basis for progestin assay, and subsequently Lutwak-Mann and Adams (3) have compared endometrial carbonic anhydrase titers and the degree of progestational proliferation as measured by the Robson (4) scale, using progesterone and other steroids. The specificity of the enzyme reaction appears to be substantiated by their data, but quantitation and definition of sensitivity were not attempted. In this paper we present: (a) a statistical analysis of the dosage response data for progesterone in the conventional Clauberg rabbit, (b) a comparison of the enzyme response with measurements of progestational proliferation, (c) data on blood carbonic anhydrase in the progesterone-treated animals, and (d) a comparative assay of progesterone and another progestin, Δ^4-pregnenolone.

MATERIAL AND METHODS

Immature albino rabbits weighing approximately 1 to 1.5 kg. were used in this study. Following the Clauberg method (5), the rabbits were injected subcutaneously once daily with 5 μg. of estradiol for six days. After this period of estrogen priming, the rabbits were injected subcutaneously once daily with the various doses of progesterone or Δ^4-pregnenolone for five days, along with 0.5 μg. of estradiol once daily for five days. The animals were sacrificed twenty-four hours after the last injection. All hormones were dissolved in Sesame oil U.S.P., and the injection volume was 0.1 to 0.2 ml. per rabbit.

The rabbits were sacrificed by breaking the neck and cutting the carotid artery. After bleeding the rabbits thoroughly, the uteri were isolated, rinsed with cold saline, and stored in the refrigerator. Small pieces of the mid-sections of both horns were cut off and preserved in Bouin's solution for histological examination, the uteri were opened longitudinally, and rinsed once with a small amount of cold saline.

To extract carbonic anhydrase the endometrium, dissected with scissors, was weighed, and put into Ten Brock homogenizers. The tissues were ground and homogenized with approximately a ten-fold volume of cold distilled water. Homogenation was always car-

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ried out in an ice-cold water bath. The homogenates were transferred into 15 ml. volumetric centrifuge tubes and centrifuged at 1500 rpm for fifteen minutes. After recording the volumes of fluid, the supernatants were decanted and used for the determination of enzyme activity.

It was confirmed that one extraction was sufficient, the residues were practically inactive, and the enzyme extracts were stable and retained their activity for several days if they were kept frozen. Distilled water was used to extract the enzyme, as it was found that hardly any differences were obtained between the activity of the enzyme extracted from the same uterus with distilled water or with very dilute Ringer-phosphate used by Lutwak-Mann (1).

The enzyme activities of the extracts were determined by the colorimetric method of Philpot and Philpot (6), one enzyme unit (E. U.) being defined as the amount of enzyme which is required to shorten the time of reaction from 65–75 seconds to 25–30 seconds (7).

It was confirmed that the activities which could be derived from the blood never exceeded one tenth of the activities of the endometrial extracts, as previously reported by Lutwak-Mann (1).

Histological preparations were made from the sections saved before extracting the enzyme, and were stained by hematoxylin-eosin. The areas of the glandular and total mucosa of the endometrium were measured with a planimeter, and G/M (glandular portion/total mucosa) was calculated by dividing the former by the latter, as described by Pincus and Werthessen (8). This ratio served as an indicator of progestational proliferation.

Two ml. of blood were taken by heart puncture. After adding 0.5 ml. of 10% sodium citrate, the bloods were diluted and laked with cold distilled water. The blood, one hundred times diluted, was used for the carbonic anhydrase assay, and the blood, two hundred times diluted, was used for the determination of hemoglobin.

In the hemoglobin assay, the densities of all samples, after removal of stroma by centrifugation, were read at optimum wave length ($\lambda = 550$ mμ) in the Coleman spectrophotometer (Model 6A). The iron content of a sample was estimated simultaneously by the colorimetric method of Wong (9). Then the hemoglobin content of each sample was calculated according to the Wong formula.

RESULTS

In Table 1 we present the mean data on carbonic anhydrase activity and glandular/total mucosal (G/M) area in a series of control and progesterone-injected animals. A linear log dose response relationship obtains over

TABLE 1. THE DOSE-RESPONSE RELATIONSHIPS FOR CARBONIC ANHYDRASE ACTIVITY AND THE G/M RATIO OF CLAUBERG RABBITS' ENDOMETRIUM

Dosage (mg/rabbit)	No. of rabbits	Carbonic anhydrase activity (EU/gm. wet tissue)	G/M ratio of uterus
0	14	17.45 ± 1.25*	0.426 ± 0.018*
0.25	6	36.30 ± 2.05	0.506 ± 0.042
0.5	9	36.14 ± 6.27	0.501 ± 0.029
1.0	6	59.67 ± 6.08	0.636 ± 0.037
2.0	10	88.05 ± 7.05	0.746 ± 0.025
4.0	12	140.03 ± 9.48	0.717 ± 0.009
8.0	6	179.57 ± 42.38	0.689 ± 0.044

EU = Enzyme unit estimated by the colorimetric method of Philpot and Philpot.
G/M = Glandular area divided by total mucosa area in uterine section.
* Mean ± S.E.

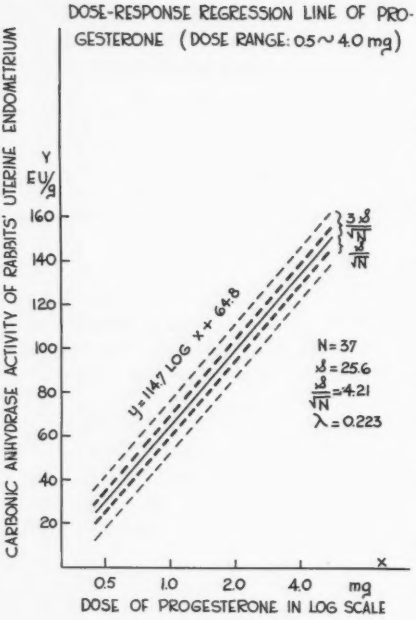

FIG. 1

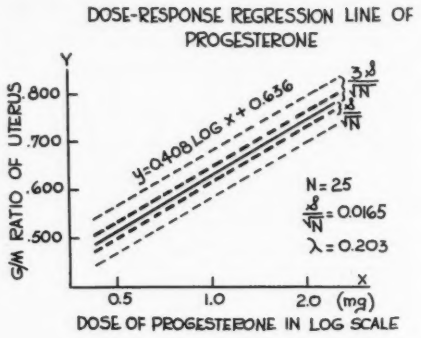

FIG. 2

the dosage range 0.5 to 4.0 mg. in carbonic anhydrase activity measurements and over the range 0.5 to 2.0 mg. for the G/M measurements. These relationships are illustrated in Figures 1 and 2. The variance analyses for these two types of measurement are presented in Tables 2 and 3. The λ values of 0.223 and 0.203 for the two types of measurement indicate comparable degrees of accuracy. Actually, a statistically significant enzyme unit:G/M correlation coefficient of 0.691 has been calculated from these data. However, over the usable concentration ranges for the two types of measurement, the enzyme concentrations exhibit an eight-fold increase over the control values, whereas the G/M measurements exhibit only a 75% increase. This greater intensity and the wider range of response in terms of enzyme activity confer useful advantages to the enzyme measurements for quantitative bioassay.

In a series of control and progesterone-injected (total dose = 4 mg.) rabbits, we have compared the carbonic anhydrase of the blood with that of the endometrium. The data are presented in Table 4. They demonstrate: (a) no significant difference in blood carbonic anhydrase between the control and progesterone-injected animals either before or after medication,

TABLE 2. ANALYSIS OF VARIANCE OF LOG DOSE-RESPONSE CURVE FOR PROGESTERONE IN FIGURE 1

Source of variation	Sum of squares	D.F.	Mean square	F_s
Slope of D/R line	59,309.42	1	59,309.42	
Variation about line	22,966.78	35	656.19	90.38
Total about mean	82,276.20	36		

$F_s = 90.38 \gg F'_{36}(0.01) = 7.39$.
Regression about the line is highly significant.
D.F. = Degree of freedom.

TABLE 3. ANALYSIS OF VARIANCE OF LOG DOSE-RESPONSE CURVE FOR PROGESTERONE IN FIGURE 2

Source of variation	Sum of squares	D.F.	Mean square	F_s
Slope of D/R line	0.28475	1	0.28475	
Variation about line	0.15747	23	0.006846	41.59
Total about mean	0.44222	24		

$F_s = 41.59 \gg F^1_{23}(\alpha) = 7.88 \quad \alpha = 0.01$
Regression about the line is highly significant.
D.F. = Degree of Freedom.

(b) highly significant endometrial increases following progesterone administration, of an order of magnitude suggesting new synthesis of enzyme in the uterus. The mean value for the control endometria is almost identical with that found by Lutwak-Mann (1).

In Tables 5 and 6, we present data on a four-point comparative assay of Δ^4-pregnenolone. Both types of measurement give entirely similar comparative potency ratios of 1.18 and 1.16, similar limits of error at $P = 0.95$. Δ^4-pregnenolone is, therefore, a progestin equal to or slightly greater than progesterone in its activity.

DISCUSSION

The data of this paper confirm the findings of Lutwak-Mann (1) that progesterone produces a significant increase in rabbit endometrial carbonic anhydrase which is proportional to the dosage of hormone administered. This increase occurs with no corresponding increase of carbonic anhydrase in the blood. Furthermore, the increase correlates significantly with the degree of uterine proliferation as measured by the G/M ratio. Finally, a comparative assay with another progestin, Δ^4-pregnenolone, demonstrates that practically identical estimates of potency may be had when either enzyme unitage or degree of progestational proliferation are used.

TABLE 4. CARBONIC ANHYDRASE ACTIVITIES OF BLOOD AND ENDOMETRIUM OF CLAUBERG RABBITS WITH AND WITHOUT PROGESTERONE TREATMENT

Treatment	No. of rabbit	Before treatment			After treatment				
		EU/ml. blood	Hb mg./ml. blood	EU/mg. Hb	EU/ml. blood	Hb mg./ml. blood	EU/mg. Hb	EU/g. Ut. endom.	G/M of uterus
Control (sesame oil)	1	533	97.4	5.48	500	89.7	5.57	18.6	0.374
	2	480	90.6	5.30	414	81.9	5.05	17.3	0.376
	3	545	97.4	5.60	511	92.5	5.52	14.5	0.405
	4	533	96.4	5.53	490	90.6	5.40	21.9	0.398
	5	522	92.5	5.64	500	89.7	5.57	15.0	0.418
	6	522	97.4	5.36	500	108.9	4.59	17.8	0.412
	Average			5.49			5.28	17.5	0.397
	S.E.			±0.05			±0.16	±1.1	±0.008
Progesterone 4 mg. (in sesame oil)	7	500	99.3	5.04	522	96.4	5.41	147.7	0.714
	8	511	90.6	5.64	490	96.4	5.08	173.0	0.750
	9	632	111.8	5.65	500	104.1	4.80	101.9	0.780
	10	444	100.3	4.43	600	94.5	6.35	137.7	0.733
	11	545	90.6	6.02	533	90.6	5.89	107.3	0.748
	12	667	103.1	6.47	500	92.5	5.41	95.6	0.728
	Average			5.54			5.49	127.2	0.742
	S.E.			±0.29			±0.23	±12.5	±0.009

EU = Enzyme units estimated by the colorimetric method of Philpot and Philpot. Hb = hemoglobin.
G/M = Glandular area divided by total mucosa area in uterine section.

TABLE 5. COMPARATIVE ASSAY OF Δ^4-PREGNENOLONE AGAINST PROGESTERONE BY ESTIMATING THE CARBONIC ANHYDRASE ACTIVITY OF CLAUBERG RABBITS' ENDOMETRIUM
(4-Point Assay)

Sample	Progesterone		Δ^4-Pregnenolone	
Dose	2 mg.	0.5 mg.	2 mg.	0.5 mg.
Response (EU/g tissue)	112.7	22.6	108.3	28.0
	118.8	71.2	76.7	50.1
	67.4	33.7	79.2	34.7
	68.8	26.7	138.1	21.7
	75.2	38.6	134.4	32.3
	81.6		116.3	
Mean	87.4	38.6	108.8	33.4

Difference between dosage: 62.1
Difference between samples: 8.1
Logarithmic ratio of dosage: 0.6
Slope of combined line: 103.44
Logarithm of potency ratio: 0.0724

$$\text{Potency ratio: } \frac{\Delta^4\text{-Pregnenolone}}{\text{Progesterone}} = \frac{1.182}{1.0}$$

Limit of error at P = 0.95: 64.9~154.2%
Parallelism of both lines is significant at P = 0.95

The usefulness of the carbonic anhydrase measurements for assay purposes resides in: (a) the intensity and range of response to varying progesterone dosages, (b) the relative ease and speed of the enzyme determination. Furthermore, presumably as the result of our use of a multiple injection regime, we have increased the sensitivity of the enzyme dose:response relationship over that previously reported (1, 2, 3) so that it is of the same order of magnitude as that of the G/M assay. Lutwak-Mann and Adams

TABLE 6. COMPARATIVE ASSAY OF Δ^4-PREGNENOLONE AGAINST PROGESTERONE BY ESTIMATING THE G/M RATIO OF CLAUBERG RABBITS' UTERI
(4-Point Assay)

Sample	Progesterone		Δ^4-Pregnenolone	
Dose	2 mg.	0.5 mg.	2 mg.	0.5 mg.
Response (G/M Ratio)	.734	.489	.733	.562
	.657	.633	.693	.585
	.711	.574	.789	.557
	.696	.515	.792	.468
	.661	.531	.688	.494
	.689		.772	
Mean	.691	.548	.745	.533

Difference between dosage: 0.1775
Difference between samples: 0.02
Logarithmic ratio of dosage: 0.6
Slope of combined line: 0.296
Logarithm of potency ratio: 0.0659

$$\text{Potency ratio: } \frac{\Delta^4\text{-Pregnenolone}}{\text{Progesterone}} = \frac{1.164}{1.0}$$

Limit of error at P = 0.95: 67.8~147.5%
Parallelism of both lines is significant at P = 0.95

(3) have demonstrated the specificity of the assay with the finding that progesterone, 17-ethinyl-19-nortestosterone, 17-hydroxyprogesterone caproate, 11-desoxycorticosterone, and 3-acetoxy-pregna-3,5-diene-20-one stimulate endometrial carbonic anhydrase increases, whereas 17-hydroxyprogesterone, 17β-acetoxy-isoprogesterone, 3α-hydroxy-pregnane-12,20-dione, 12α-hydroxy-pregnane-3,20-dione, pregnane-3,12,20-trione, 2β-acetoxy-progesterone, 2α-acetoxy-progesterone, and pregna-5-ene-20-one do not. We shall report later similar studies with a variety of steroids, but it suffices here to point out that with the development of a method susceptible to conventional dosage:response analysis, more exact definitions of comparative potencies are made possible, as well as departures from the dosage:response relationship observed with certain compounds.

SUMMARY

The uterine mucosa of fourteen control and forty-nine progesterone-treated, immature, estrogen-primed rabbits were analyzed for carbonic anhydrase content and degree of progestational proliferation (G/M ratio). Significant log dose:response relationships of similar degrees of accuracy were obtained with either measurement, but over the range 0–4 mg. for the carbonic anhydrase and over the range 0–2 mg. for the G/M ratio. The increase in mucosal carbonic anhydrase during progesterone administration was not accompanied by any increase in blood carbonic anhydrase. A comparative assay of Δ^4-pregnenolone, using progesterone as a standard, indicated a potency ratio to the standard of 1.18 by the carbonic anhydrase method and of 1.16 by the G/M ratio method, suggesting the usefulness of the carbonic anhydrase method as a standard assay procedure.

Addendum

After this paper was written, we have observed that a little greater intensity of response to progesterone can be obtained in the enzyme determination by omitting the 0.5 μg. of estradiol which was injected once daily with the progesterone. Therefore, the administration of estradiol after estrogen priming should be omitted in order to get the greater response to progesterone, although the estrogen priming may be necessary to make it easier to dissect off the endometrium.

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IN VITRO ACTION OF THYROXINE ANALOGS ON SUCCINATE AND MALATE OXIDATION[1]

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IN THE search for an explanation of how thyroxine acts to increase the rate of oxygen consumption of various tissues of the body, many workers have examined enzyme effects (1). Considering that the activity of several enzyme systems, such as succinoxidase, cytochrome C and cytochrome oxidase, is appropriately increased in hyperthyroidism, it is logical to attempt to demonstrate direct hormonal effects on these. In general, Smith and Williams-Ashman (2) and others found no consistent stimulation. This may well be due to the fact that enzyme studies are carried out under conditions so chosen as to yield maximal activities. With less favorable conditions, thyroxine might act to prevent denaturation of specific proteins, to stabilize oxidation-reduction potentials, etc. Such an action may not even remotely be related to the *in vivo* effect of the hormone.

Thus, the thyroxine potentiation of the ascorbic acid oxidase system, pointed out by Gemmill (3), was shown by Frieden (4) to be a protective action shared by gelatin and by various amino acids, including one with considerable thyroxine-inhibiting power *in vivo*.

Although no thyroxine alteration, other than inhibition at high concentrations, is obtained on the succinoxidase determination as usually carried out, when a high concentration of phosphate is present, the hormone has a marked enhancing effect (5, 6). This was confirmed by Estabrook *et al.* (7), who were able to duplicate the effect with added calcium salts. These workers suggested that thyroxine and calcium both might lower the accumulation of oxaloacetate, a potent inhibitor of succinic dehydrogenase. This surmise was proven by Clarke and Ball (8), who placed the site of thyroxine action at the malic dehydrogenase enzyme responsible for oxaloacetate production:

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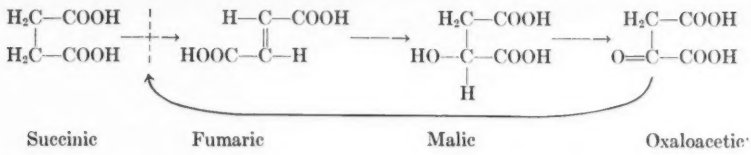
It is a pleasure to acknowledge the generosity of the following persons in furnishing thyroxine analogs: Dr. R. Pitt-Rivers, National Institute for Medical Research, London: Dr. J. A. King of Warner-Chilcott Laboratories: Dr. H. A. Fevold of Travenol Laboratories: Dr. A. E. Heming, Smith, Kline and French Laboratories: Dr. Stanley Wawzonek of the State University of Iowa.



Wolff and Wolff (9) demonstrated that the purified malic dehydrogenase enzyme was inhibited by thyroxine and some related compounds.

Our interest in this thyroxine effect was to find out whether the action could be correlated in any way with the effect of the hormone and its analogs on energy metabolism.

EXPERIMENTAL METHODS

Rats of the Sprague-Dawley strain were exsanguinated from the abdominal aorta under ether anesthesia. Tissues to be used were quickly removed and placed in cold 0.9% NaCl solution. Portions of tissue suitable for homogenization were prepared, blotted and rapidly weighed on a torsion balance. Grinding was achieved in the Potter-Elvehjem glass apparatus (10) with sufficient 0.1 *M* phosphate buffer of pH 7.8 to yield a 6.7% w/v suspension. Every possible attempt was made to obtain a consistent homogenization, keeping the tubes in an ice-bath. The suspension was rapidly added to the previously prepared and cooled Warburg vessels with a wide-tip pipette.

The actual composition of the system, modified from Wiswell *et al.* (6), was as follows:

0.8 ml. 1.0 *M* phosphate buffer, pH 7.8
0.3 ml. 0.005 *N* NaOH containing thyroxine or analog, 3×10^{-4} *M*
0.2 ml. 0.8 m *M* cytochrome c in 0.08 *M* NaCl solution
0.4 ml. heart homogenate; 1 gm. tissue plus 14 ml. 0.1 *M* phosphate buffer, pH 7.8
1.1 ml. water
0.2 ml. 0.31 *M* sodium succinate (8.4% of the salt with 6 H_2O) in side arm; for sodium malate, 41.5 mg. of malic acid are used per ml. of solution with enough NaOH to bring the pH to 7.8.

Since thyroxine and nearly all of its analogs tested require approximately 0.005N NaOH to be held in solution, the control flasks received an equivalent quantity of the alkali alone. With a total volume of 3.0 ml., the final concentration of thyroxine or analog was 3×10^{-5} *M*.

The substrate, either succinate or malate, was tipped into each vessel just prior to the start reading, after a fifteen-minute period at 37° for temperature equilibrium. Readings were taken at fifteen minute intervals thereafter, without interrupting the shaking, for 60 to 120 minutes.

RESULTS

Figure 1 presents the rat heart homogenate succinoxidase activity over two hours both in the absence and presence of thyroxine, computed as μl O_2 consumed per mg. tissue homogenate per hour (corresponding to the customary "Q_{O_2}" value of tissue metabolism). It is quite obvious that both the thyroxine and control flasks fell off rapidly with time. In spite of the higher values for the thyroxine, the rapid change made it necessary to pick an arbitrary length of time for comparison. Since the greatest amount of activity in both blank and thyroxine sets of vessels took place from 0 to 30 minutes, this time limit was consistently adopted. Calculations using

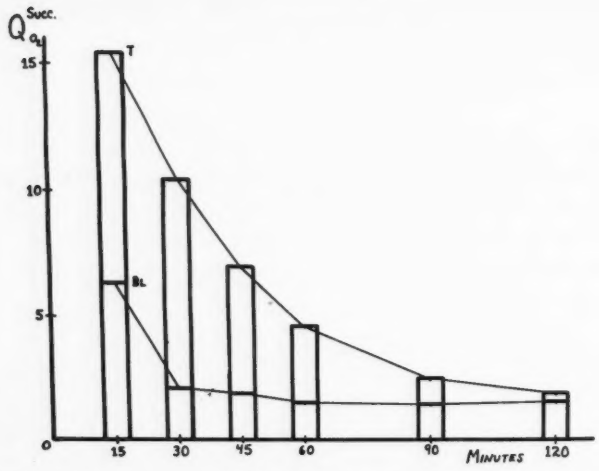


FIG. 1. Greater succinoxidase activity of rat heart homogenate in the presence of thyroxine (T) than in control (BL). Note rapid fall in activity over the first 60 minutes. For details of the procedures used in this and other figures, the text should be consulted. (Reproduced from Vol. 10, Ciba Foundation Colloquia on Endocrinology, 1956, by permission of the editors and Messrs. J. & A. Churchill, London, publishers.)

the 0–60 minute values were also made and comparison showed no marked differences in interpretation.

The data for succinoxidase effects of thyroxine and four of its analogs obtained in this manner are graphed in Figure 2, with thyroxine at the far left. Considering the control flasks (with blank solution instead of thyroxine) as 100%, the thyroxine values averaged 276%. Although this is a clear-cut and consistent effect, it is difficult to defend rigorously an interpretation as a true stimulation, in view of the marked falling off.

For comparison of other analogs with thyroxine, the Q_{O_2} value of 7.5 above the control furnishes a convenient denominator. On this basis, the tetraiodo-thioether analog exerted 95% as much effect as thyroxine, the triiodothioether 87%, the tetrabromo 61% and the tetrachloro 9%.

From Figure 3 it can be seen that oxygen consumption in the presence of malate as substrate ("malic oxidase") also fell off rapidly during the first hour of study. In the presence of thyroxine, malic oxidase activity was depressed from the beginning. This confirms the work of Clarke and Ball along the same lines, and strongly supports their concept that the thyroxine "stimulation" of succinoxidase occurred because the hormone prevented accumulation of oxaloacetate from malic dehydrogenase action.

Further support for this thesis is seen after the addition of glutamate, which removes oxaloacetate by transamination to form aspartate and α-ketoglutarate, neither of which interferes with succinoxidase. Figure 4

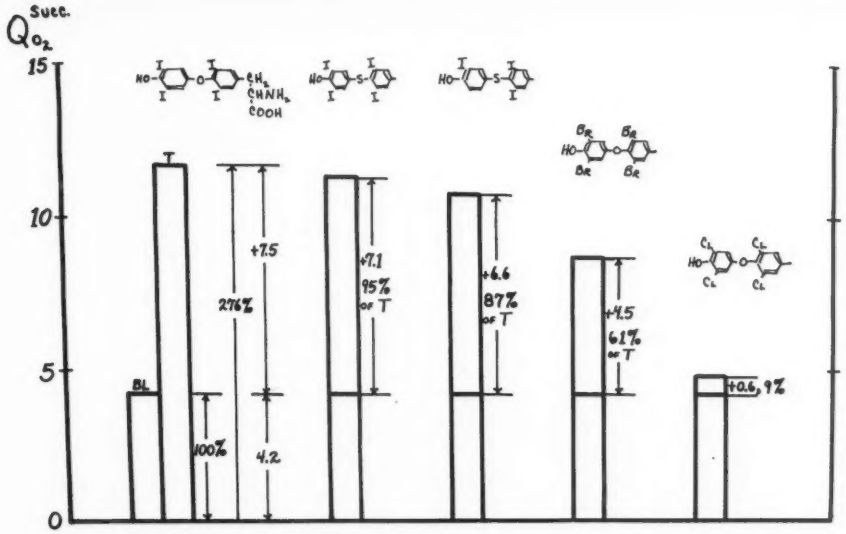


FIG. 2. Comparison of 30-minute effect on heart succinoxidase of five thyroxine analogs with thyroxine itself (far left). The calculated 276% effect of thyroxine (T) is shown graphically as compared with the control (BL) enzyme activity. In addition, the basis for a quantitative evaluation of each analog is pictured. The compounds are, from left to right: thyroxine, thioether analog of thyroxine, thioether analog of triiodothyronine, tetrabromothyronine and tetrachlorothyronine.

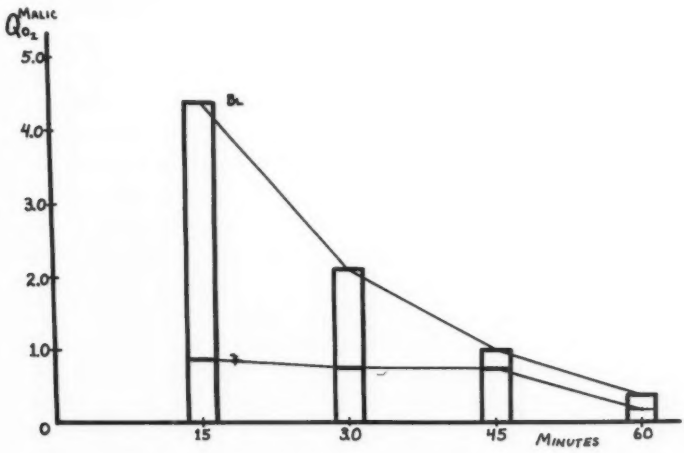


FIG. 3. Inhibition of malic acid oxidation by rat heart homogenate in the presence of thyroxine (T) below control activity (BL). (Reproduced from Vol. 10, Ciba Foundation Colloquia on Endocrinology, 1956, by permission of the editors and Messrs. J. &. A. Churchill, London, publishers.)

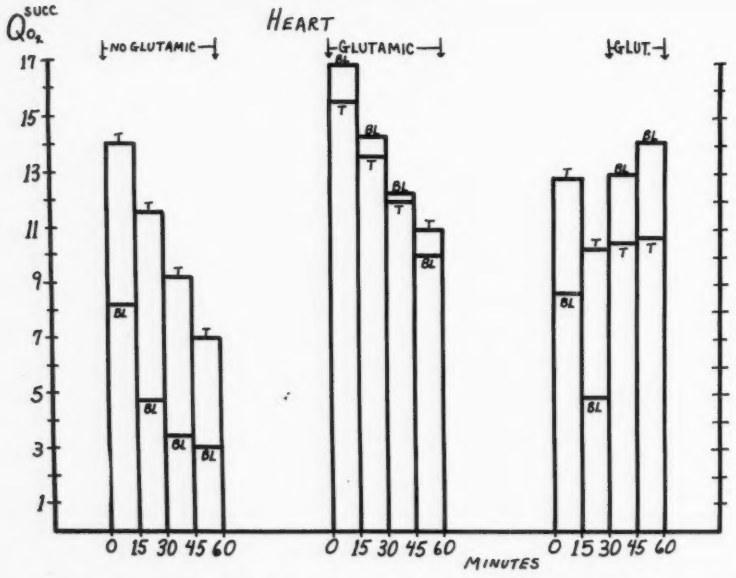


FIG. 4. Effect of addition of glutamate on oxidation of succinate by rat heart homogenate. Note that the presence of glutamate from the start of the experiment (center series) eliminates the thyroxine enhancement. On the right, it is shown that, after thyroxine has produced greater activity for 30 minutes, addition of glutamate raises the control up to the original thyroxine-activated level.

demonstrates that addition of glutamate at the start of the succinoxidase run completely abolished the thyroxine effect. When the glutamate was not added until after 30 minutes, the thyroxine effect was promptly reversed at that point, and the control values raised to the starting thyroxine level. Parenthetically, it may be noted that similar confirmation of the Clarke and Ball effect has been obtained for diaphragm (Fig. 5), but that liver succinoxidase, although responding well to thyroxine, is not altered by glutamate added at any time during the experiment (Fig. 6). This particular point will not be pursued further in this paper, although it is deserving of further attention.

The left-hand portion of Figure 7 shows the action of thyroxine depressing malate oxidation 73% from the control 30-minute level, the counterpart of the succinoxidase elevation. Using the calculated drop in Q_{O_2} of 1.92, the capacity of each of the same four analogs to depress malate oxidation can be computed in terms of the thyroxine effect. By comparison with Figure 2, it can be seen that the enhancement of succinoxidase corresponded fairly well with inhibition of malic oxidase in each case.

In order to facilitate this comparison, Table 1 contains the structures of a variety of compounds tested together with their effects on succinate and

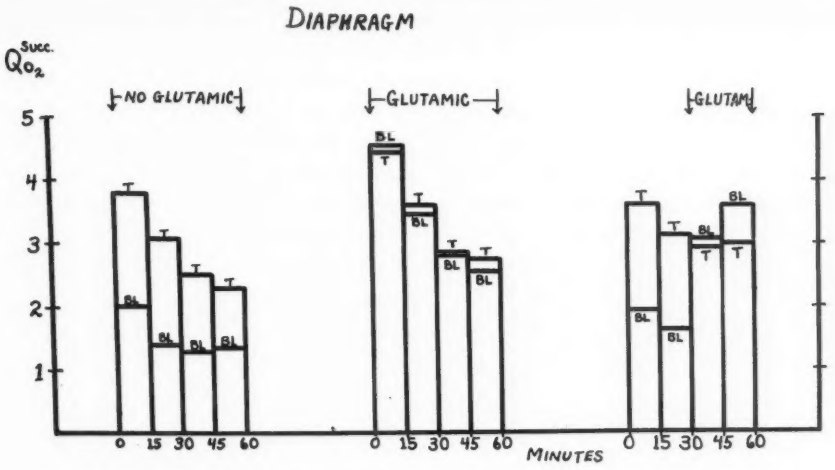


FIG. 5. Effect of addition of glutamate on oxidation of succinate by rat diaphragm homogenate. The comments made on Figure 4 for heart also apply here.

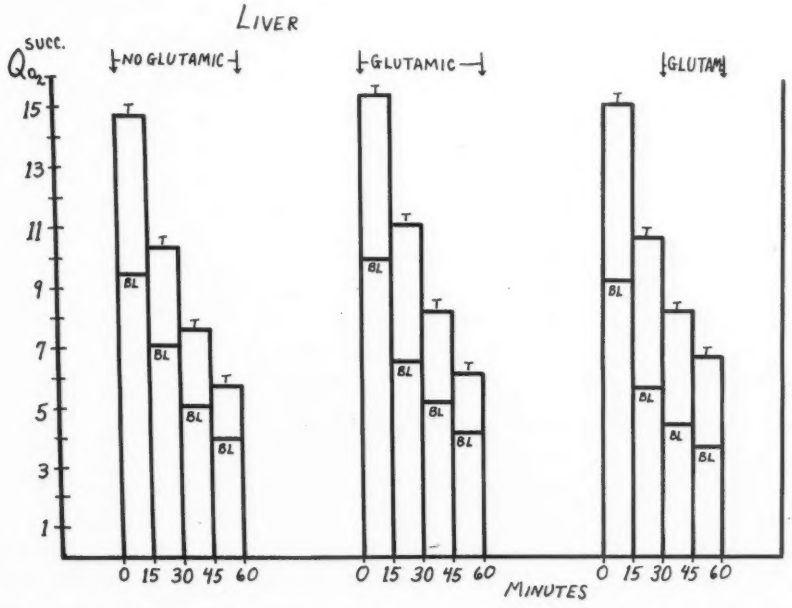


FIG. 6. Lack of effect of glutamate on oxidation of succinate by rat liver homogenate. In contrast to heart and diaphragm, the addition of glutamate at the start of the experiment or 30 minutes later produced no alteration. This indicates a difference in the mechanism of the thyroxine-enhanced succinoxidase activity in liver from that in heart or diaphragm.

malate oxidation. With only minor discrepancies, the substances with over 50% as much activity as thyroxine on succinate possessed corresponding (reversed) action on malate. However, tribromothyronine, 2,4-diiodophenoxyacetic acid and diiodothyronine all inhibited malate to a considerably greater extent than they stimulated succinate. Several of the 7 analogs without appreciable effect on succinoxidase exhibited considerable malate effect, especially Amiodoxyl benzoate, 2,6-diiodophenoxyacetic acid and the *n*-butyl ester of 3,5-diiodo-4-hydroxybenzoic acid. One can

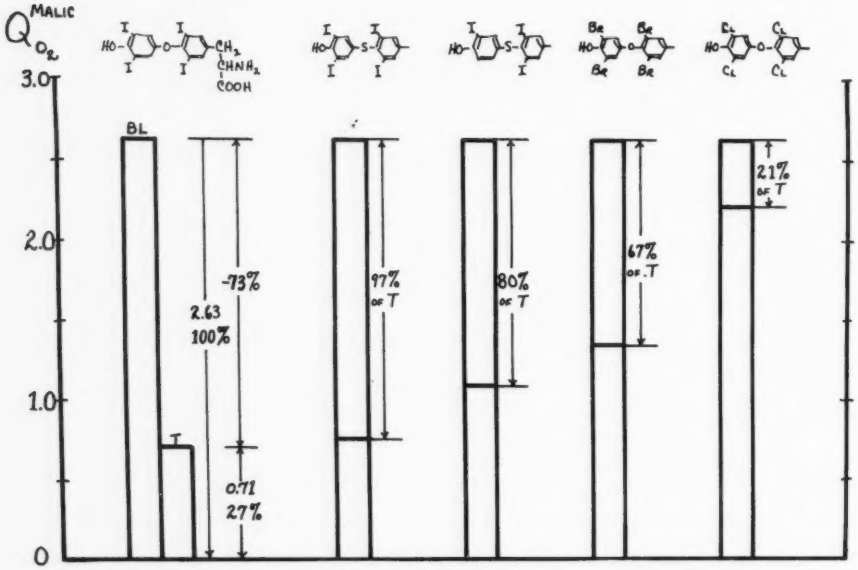


FIG. 7. Comparison of 30-minute effect on heart malic oxidation of five thyroxine analogs with thyroxine itself (far left). The calculated 73% inhibition of thyroxine (T) is shown graphically. As in Figure 2, the basis for quantitative evaluation of the same analogs is pictured.

only assume that these compounds also have a depressant action directly on the succinoxidase itself.

Thus, the theory advanced by Clarke and Ball and further extended by Wolff and Ball (9a) seems well founded, that the thyroxine "stimulation" of succinoxidase activity is based on prevention of the accumulation of oxaloacetate. Inspection of structural requirements reveals some interesting relationships. Among the top eighteen compounds, all with activities on succinoxidase and malic oxidase within 60% as active as thyroxine itself, there are two with no halogens on the outer ring (3,5-diiodothyroacrylic and 3,5-diiodothyropropionic). Furthermore, five others, 3,5-diiodo-3′5′-dibromothyronine, 3,5-diiodo-3′,5′-dichlorothyronine, 3,5-di-

TABLE 1. COMPARISON OF *In Vivo* AND *In Vitro* EFFECTS OF THYROXINE ANALOGS

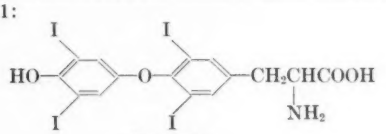
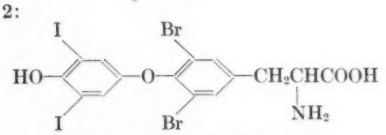
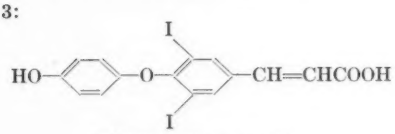
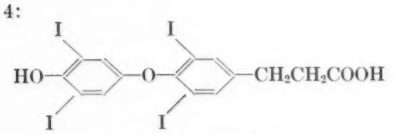
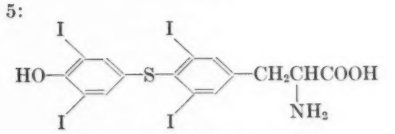
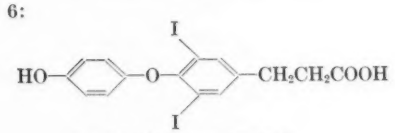
Compound	Per cent of thyroxine activity			
	In Vitro		*In Vivo**	
	Succinate	Malate	Value	Technic
1:  Thyroxine	100	100	100	
2:  3,5-Dibromo-3′,5′-diiodothyronine	107	90	12.5 12.5	Metabolism of thyroidectomized rat (16) Anti-goitrogenic (17)
3:  3,5-Diiodo-thyroacrylic	101	87	3	Metabolism of thyroidectomized rat (18)
4:  Tetraiodo-thyropropionic	96	94	73	Metabolism of thyroidectomized rat (18)
5:  Thio-ether analog of thyroxine	95	97	11	BMR of myxedema (19)
6:  3,5-Diiodo-thyropropionic	95	76	2.5	Metabolism of thyroidectomized rat (18)

TABLE I (*continued*)

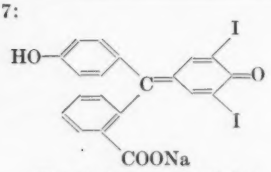
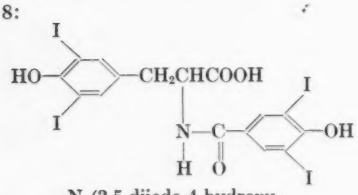
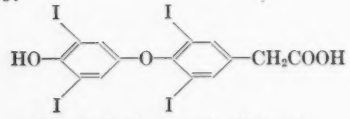
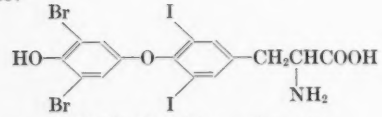
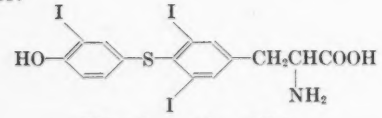
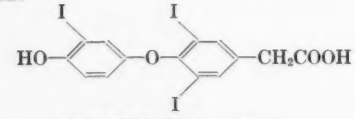
Compound	Per cent of thyroxine activity			
	In Vitro		*In Vivo**	
	Succinate	Malate	Value	Technic
7:  Tetraiodo-phenolphthalein	95	90	0	Metabolism of thyroidectomized rat (18)
8:  N-(3,5-diiodo-4-hydroxybenzoyl)-3,5-diiodo-tyrosine (B-5522)	94	94	0	Metabolism of thyroidtomized rat (18)
9:  Tetraiodo-thyroacetic (Tetrac)	94	97	76	Metabolism of thyroidectomized rat (18)
10:  3,5-Diiodo-3′,5′-dibromothyronine	92	98	10 10	Metabolism of thyroidectomized rat (16) Antigoitrogenic (17)
11:  Thio-ether analog of Trit	88	80		
12:  3,5,3′-Triiodo-thyroacetic (Triac)	86	95	117	Metabolism of thyriodectomized rat (18)

TABLE I (*continued*)

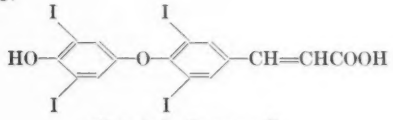
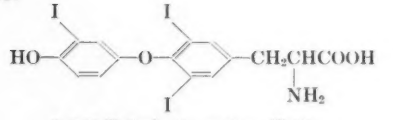
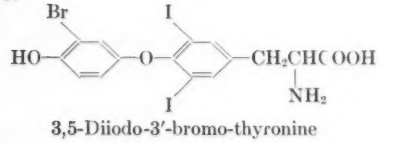
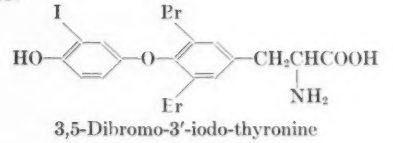
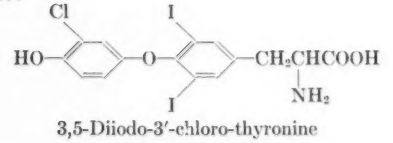
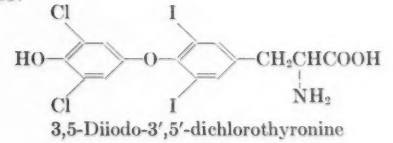
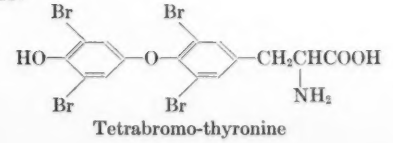
Compound	Per cent of thyroxine activity			
	In Vitro		*In Vivo**	
	Succinate	Malate	Value	Technic
13:  Tetraiodo-thyroacrylic	83	109	76	Metabolism of thyroidectomized rat (18)
14:  3,5,3′-Triiodo-thyronine (Trit)	82	90	420 310	Metabolism of thyroidectomized rat (18) Antigoitrogenic (17)
15:  3,5-Diiodo-3′-bromo-thyronine	81	92	200	Antigoitrogenic (17)
16:  3,5-Dibromo-3′-iodo-thyronine	79	94	130	Antigoitrogenic (17)
17:  3,5-Diiodo-3′-chloro-thyronine	79	59		
18:  3,5-Diiodo-3′,5′-dichlorothyronine	76	89	0.0	Metabolism of thyroidectomized rat (16)
19:  Tetrabromo-thyronine	61	67	0.02 0.5	Metabolism of thyroidectomized rat (16) Antigoitrogenic (17)

TABLE I (*continued*)

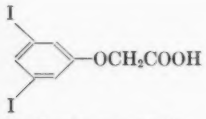
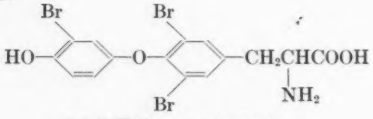
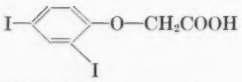
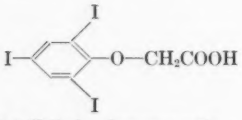
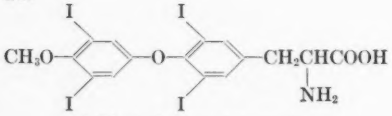
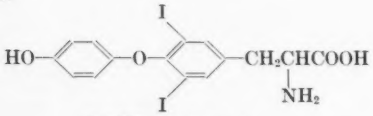
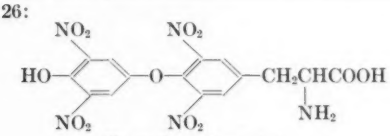
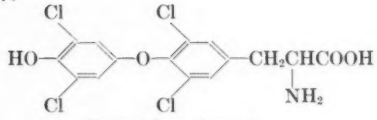
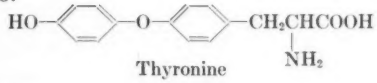
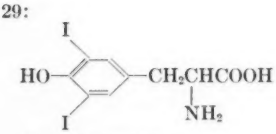
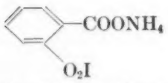
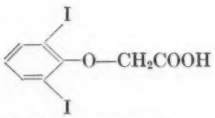
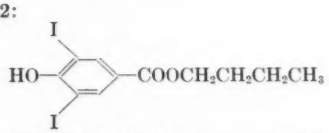
Compound	Per cent of thyroxine activity			
	In Vitro		*In Vivo**	
	Succinate	Malate	Value	Technic
20:  3,5-Diiodo-phenoxyacetic	48	41	0.0	Metabolism of thyroidectomized rat (18)
21:  3,5,3′-Tribromo-thyronine	46	71	44	Antigoitrogenic (17)
22:  2,4-Diiodo-phenoxyacetic	39	59	0	Metabolism of thyroidectomized rat (18)
23:  2,4,6-Triiodo-phenoxyacetic	34	38	0	Metabolism of thyroidectomized rat (18)
24:  O-Methyl ether of thyroxine	34	31	33 5	Metabolism of normal rats (20) Antigoitrogenic (20)
25:  3,5-Diiodo-thyronine	30	63	0.0	Metabolism of thyroidectomized rat (18)

TABLE I (*continued*)

Compound	Per cent of thyroxine activity			
	In Vitro		*In Vivo**	
	Succinate	Malate	Value	Technic
26:  Tetranitro-thyronine	9	9	1.0	Metabolism of thyroidectomized rat (21)
27:  Tetrachloro-thyronine	8	21	0.0	BMR of myxedema (19)
28:  Thyronine	4	—3	0.0	Metabolism of thyroidectomized rat (18)
29:  Diiodo-tyrosine	0	23	0.0	Metabolism of thyroidectomized rat (18)
30:  Ammonium-1-iodoxy-benzoate	16	64	0.0	Metabolism of thyroidectomized rat (18)
31:  2,6-Diiodo-phenoxyacetic	—6	64	0.0	Metabolism of thyroidectomized rat (18)
32:  n-Butyl-3,5-diiodo-4-hydroxy-benzoate	—7	60	0.0	Metabolism of thyroidectomized rat (18)

* Only a few figures are given here, for the sake of comparison. For an exhaustive summary of this literature, the review of Selenkow and Asper (15) should be consulted.

iodo-3′-bromothyronine, 3,5-diiodo-3′-chlorothyronine and tetrabromothyronine, contain halogens with far less activity than iodine on the outer ring. The tetraiodophenolphthalein and N-(3,5-diiodo-4-hydroxybenzoyl)-3,5-diiodotyrosine ("B-5522") have no diphenyl ether linkage. Some of the compounds are fatty acid derivatives rather than amino acids.

Two interesting members of this series, the tetraiodophenolphthalein and B-5522, were compared with thyroxine using a highly purified malate-malic dehydrogenase system, similar to that studied by Wolff and Wolff (9). Concentrations of 22 and 33 μg./ml., respectively, were required for a 50% inhibition of the enzyme, compared to 21 μg./ml. for thyroxine. This is further evidence that these widely varying structures are able to produce similar effects.

All in all, it seems reasonable to conclude that the structure most closely correlated with the particular enzyme activities being discussed here is one containing two benzene rings, at least one of which should have the 3,5-diiodo-4-hydroxy configuration. Scrutiny of the other compounds exhibiting some, but less, activity reveals that bromine may substitute for iodine, but that chlorine is essentially inactive.

DISCUSSION

The conclusions reached above regarding structural requirements for succinoxidase and malic oxidase activities amongst thyroxine analogs suggest that an even poorer correlation exists between these enzyme functions and *in vivo* metabolic activity. A more detailed examination of Table 1 brings this out very clearly. For some of these compounds, metabolic data are lacking, but using goiter-prevention data in these instances, nine of the eighteen most active on enzyme function were less than 15% as active metabolically as thyroxine. Two of these, tetraiodophenolphthalein and the B-5522, have no activity whatsoever. In fact, the latter displays definite thyroxine-antagonistic action (11).

This brings out a very important point which should be stressed: just because a compound, in this case thyroxine, is capable of producing an effect on an enzyme system *in vitro* which appears qualitatively similar to an *in vivo* change, it does not prove that this is actually the mechanism of operation of the hormone. This is not to suggest that such studies should not be carried out; truly, the more information obtained about any effect of thyroxine, the closer is a genuine understanding of the hormone's mode of action in the body. However, a great deal of caution obviously must be exercised in bridging such large gaps as that which still exists between biological material at the "intact cell" level and at the enzyme. In general, studies of the latter determine fundamental potentialities, but, by their very nature, cannot reveal quantitative relationships of these same systems under *in vivo* conditions of intact cellular organization.

It is appropriate to comment further that, in the search for *in vitro* hor-

monal responses, absolute quantitative correspondence may not always be obtained. In the present series, for instance, T3 (3,5,3′-triiodothyronine, commonly referred to as "triiodothyronine"), is by no means the most active analog tested, although it is several times more active *in vivo* than any other substance. Actually, its succinic and malic oxidase effects are somewhat greater during the first fifteen minutes, and then fall off more rapidly than the others, a situation similar to its metabolic action *in vivo*. A study of such quantitative differences may eventually uncover a genuine clue to explain the greater activity of T3 over T4 *in vivo*. This is far less critical in rejecting an *in vitro* enzyme effect as the demonstration of activity equal to thyroxine on the part of tetraiodophenolphthalein and B-5522. Considering such an enhancement from these compounds which do not directly alter metabolism, it is virtually impossible to conceive of this enzyme mechanism playing a significant role in the evolution of a metabolic response.

The need for a thoroughly critical examination of all *in vitro* thyroid responses is indicated by certain other responses, such as the ascorbic acid oxidase already discussed, and also the increase in Ehrlich ascites tumor cell glycolysis. It was originally suggested by Heimberg *et al.* (12) that this latter reaction was characteristic of the tetraiodo- and triiodothyroacetic derivatives. However, Barker and Lewis (13) clearly demonstrated a lack of specificity, with B-5522 showing good responses, as well as thyroxine, T3 and the acetic acid analogs.

There is, in fact, even more important a discrepancy between *in vitro* and *in vivo* action which is not immediately apparent from the present work. This is, that if increased overall metabolism is dependent upon increased oxidation of succinate, increased amounts of fumarate and malate will probably be presented for metabolism. If thyroxine can stimulate oxidation of succinate only by inhibiting that of malate, a fundamentally paradoxical situation is obtained, which is of no value in helping to understand the physiological role of thyroxine. Adequate attention has not previously been paid to this situation.

In order not to leave the discussion on a purely negative note, it is well to mention a kidney slice incubation technic involving an *in vitro* metabolic response of intact cells to thyroxine (14). Preliminary testing with several analogs shows a high degree of biochemical specificity. Using this approach, it is hoped to elucidate more of the action of thyroxine than has been possible before.

SUMMARY

This paper has presented a critical discussion of *in vitro* responses to thyroxine, especially of their supposed relation to *in vivo* mechanism of action of the hormone.

The point is stressed that for an *in vitro* response to be of value in inter-

preting *in vivo* function, it must be specific at least to the extent of not being altered by thyroxine analogs which are inactive *in vivo*. This is here illustrated by the *in vitro* thyroxine enhancement of succinoxidase and depression of malic dehydrogenase. Even if thyroxine's metabolic action *in vivo* should depend upon an increased succinoxidase activity, it cannot act this way for two reasons: first, thyroxine analogs which have no metabolism-stimulating action in the intact animal produce as much effect on enhancing succinate oxidation and in depressing malate oxidation. Secondly, if thyroxine is to stimulate oxidations in an overall fashion, a highly unphysiological situation would be presented by a succinoxidase stimulated only by a depression in malic dehydrogenase, since it should be necessary for the body to handle increased amounts of malate resulting from the oxidation of succinate.

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THYROTROPHIN POTENCY OF TRANSPLANTABLE PITUITARY TUMORS OF MICE THROUGH FOUR TRANSFERS

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IN RECENT years, several different types of transplantable, functional pituitary tumors have been developed in mice and rats (1 and 2). Most remarkable among these are the transplantable thyrotrophic pituitary tumors which can be induced by radiothyroidectomy, surgical thyroidectomy, or blocking of thyroid hormonal synthesis by anti-thyroidal compounds. Earlier studies have shown that the thyrotrophic tumors have a high hormonal content. Most studies, to date, have, however, been of the physiological nature of the tumors and histological techniques have been used to describe the tissue changes that occurred. The following study for the first time records weight changes in various endocrine organs and gives quantitative values for the concentration of thyrotrophin in the tumors and the blood of the mice through successive generations of transplantation.

MATERIAL AND METHODS

Mice: All mice used were of the C57Bl. strain. After 2–3 weeks on a low iodine diet they were completely radiothyroidectomized by injection of 100–150 μc. of I^{131}.

Tumor Strains: All tumor strains described here originated in the laboratories of Furth and associates. The primary tumors were the enlarged pituitaries from radiothyroidectomized mice. Each strain had been transferred for several generations before the generation designated "O" in this paper, which refers to the beginning of this study at the National Institutes of Health (NIH). Autonomy developed in one line of strain 77D in the fifth subpassage and in one line of strain 101D in the third subpassage in Furth's laboratories. At NIH the strains have remained dependent upon thyroidectomized hosts.

Tumor Generations: The numbers designate the transfer generation. 2A refers to a transplantation from an early tumor of generation 1 at 6.5 months, and 2B refers to transplantation from a slow growing or late tumor of generation 1 at 11.2 months.

Tumor Transplantation: A freshly harvested tumor was cut into pieces which were further broken up gently by hand in a Potter type homogenizer using 3 to 5 volumes of physiological saline. With the use of an 18 gauge needle, 0.2 ml. of the suspension was injected subcutaneously or intramuscularly into one hind leg. The implanted mice were examined for tumors every 2–3 weeks thereafter.

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Bioassay of TSH in Blood and Tumors: When the tumors reached a weight of about 5 grams, blood was collected for TSH assay, the mice were killed and the tumors harvested. The excised tumors were frozen at once on dry ice and kept in a home freezer until lyophilized. The dry tumor was ground to 40 mesh size in a Wiley mill. For bioassay, this powder was injected as a suspension in 0.1 M sodium bicarbonate. Heparinized blood was collected from the abdominal aorta before autopsy. The red cells were removed by centrifugation and the plasma collected and stored frozen. The fibrin clot, which forms upon thawing, was removed.

The concentration of TSH was determined by the method of Bates and Cornfield (3), using sufficient animals so that the accuracy was ±30%. All potencies are in terms of U.S.P. units of thyrotrophin.

RESULTS AND CONCLUSIONS

Course of Tumor Growth: The course of tumor growth was similar in the three tumor strains, but the growth rate differed (Fig. 1). Strain 447D showed palpable tumors after 2 to 3 months. Some of these tumors were large enough to harvest after 4 months at about the time when tumors of Strain 101D were barely palpable. As a result of this difference in growth rate, there have been as many generations of 447D in a little over 2 years as of 101D in 4 years. Strain 77D is intermediate in growth rate. In all the strains the tumor grows to a size of 3 to 5 grams in weight in about 2 months after reaching palpable size, more or less independently of the "latent" period of growth. Within each strain there has been no significant change in rate of growth in the four transfer generations. A difference in

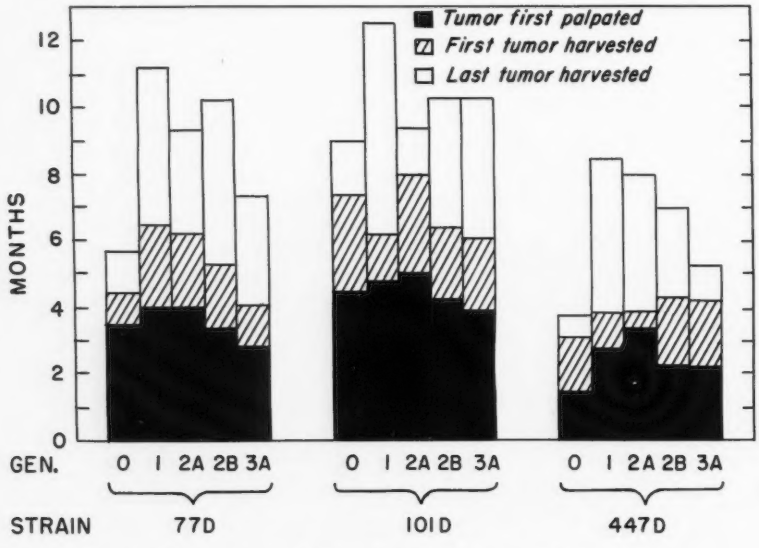

FIG. 1. Time course of tumor growth in three strains of tumor.

TABLE 1. ORGAN WEIGHTS OF RADIOTHYROIDECTOMIZED MALE MICE WITH TRANSPLANTABLE THYROTROPHIC PITUITARY TUMORS

Tumor		No. mice	Body wt., gm.	Testes wt., gm.	Seminal vesicle wt., mg.	Adrenal wt., mg.	Pituitary wt., mg.	Large bile duct %
Strain	Generation							
77D	1	17	30.6±0.8*	173± 4	157±13	5.5±0.2	3.4±0.6	47
	2A	26	33.7±0.5	180± 3	114± 9	6.1±0.1	2.9±0.1	8
	2B	9	33.8±0.2	161± 9	102±15	5.6±0.2	2.9±0.5	11
	3A	8	32.3±1.0	185± 4	119±16	6.2±0.2	3.1±0.3	0
101D	0	22	31.2±0.4	162± 4	79± 6	5.5±0.2	2.9±0.5	0
	1	11	24.8±1.3	135± 9	109±15	5.3±0.4	27.5±8	0
	2A	25	28.5±0.7	163± 4	118± 6	4.9±0.1	5.5±1	0
	2B	5	30.8±0.8	165± 8	94±10	5.2±0.6	2.9±0.2	20
	3A	9	31.0±1	168± 8	114±12	5.2±0.2	11.8±8	0
447D	0	4	29.5±1.8	170±10	171± 7	6.6±0.7	1.6±0.5	75
	1	9	31.4±1.0	164± 8	134± 1	5.7±0.2	2.7±0.9	22
	2A	5	32.4±1.2	167±13	111±18	5.4±0.3	2.4±0.2	0
	2B	18	28.0±1.3	—	—	—	—	0
No tumor —		6	27±0.8	160± 8	118±29	5.8±0.4	12.8±3.4	0
Normal mice		29	28±0.5	156± 5	170±11	4.6±0.2	1.7±0.1	0

* Standard error.

the age of tumor of 4–5 months at the time of transfer (2A vs. 2B generations) does not appear to alter the course of tumor growth.

Weights of Endocrine Organs: Organ weights indicate the blood level of hormones that stimulate the organ even when that level is too low to determine by direct bioassay procedures using blood. In Table 1 and 2 it is seen that most tumor bearing animals weigh 4 to 5 grams more than control mice which are without tumors. The weight of the tumor (about 5 gm.) accounts for this weight difference. There is no evidence for growth hormone production by the tumor.

Pituitary gland weights are independent of the tumor strain. The increased weight of the pituitary glands of the host are related to the length of time after radiothyroidectomy. Hence, the pituitaries were not much enlarged in the mice bearing tumors of rapidly growing strain 447D and were larger in mice with tumors of the slow growing strain 101D. The pituitaries of the control radiothyroidectomized mice were largest because the mice were not killed until about 1 year after thyroidectomy at which time most athyroid mice have either primary microtumors or small gross tumors as a consequence of uncompensated deficiency of thyroid hormone (4).

Adrenal gland weights which vary in an irregular manner give no significant indication that ACTH is produced by the tumors.

In male mice there is no consistent evidence from weights of testes or seminal vesicles that the tumors are producing gonadotrophins, but in

TABLE 2. ORGAN WEIGHTS OF RADIOTHYROIDECTOMIZED FEMALE MICE WITH TRANSPLANTABLE THYROTROPHIC PITUITARY TUMORS

Tumor		No. mice	Body wt. gm.	Ovary wt., mg.	Uterus wt., mg.	Adrenal wt., mg.	Pituitary wt., mg.	Large bile duct %
Strain	Generation							
77D	0	31	29.3±0.8*	35±5	219±24	8.3±0.4	4.0±0.3	45
	1	17	28.4±0.6	19.1±2	168± 9	5.8±0.1	4.2±0.2	53
	2A	4	24.4±0.2	20.2±12	164±22	5.5±0.5	6.1±2.7	0
	2B	18	29.8±0.4	48.7±12	205±16	6.9±0.3	3.7±0.2	61
	3A	7	30.3±0.3	56.1±10	191±17	10.0±0.9	3.6±0.1	71
101D	1	15	26.8±0.9	6.7±1.3	120±11	5.7±0.2	5.5±0.5	0
	2A	5	27.6±1.5	10.7±3	200± 3	5.7±0.4	5.0±1.0	20
	2B	15	29.1±0.7	9.0±1.6	148±13	5.9±0.2	3.6±0.1	0
	3A	9	28.6±1.0	10.5±1.9	159±14	6.7±0.2	4.8±0.6	22
447D	1	9	28.8±0.7	12.8±1.5	208±11	6.4±0.2	2.9±0.2	100
	2A	16	28.0±1.3	14.9±2.4	166±34	6.9±0.4	2.7±0.3	31
	2B	10	27.6±1.2	—	—	—	—	30
No tumor	—	4	23.6±1.2	4.8±1.2	55±23	5.3±0.7	15.6±5.2	0
Normal mice		45	24.9±0.2	3.4±0.3	86± 5	6.1±0.2	2.1±0.1	0

* Standard error.

female mice, especially in strain 77D, there is overwhelming evidence from both ovarian and uterine weight of production of gonadotrophin by the tumor. With all strains of tumor the ovaries are large and contain many hemorrhagic follicles; the uterus is usually distended with fluid, which was removed before weighing; females are in practically constant estrus; the pubic bones are 1–2 mm. apart and the pelvis readily flexible. These sex organ effects in the female and not in the male suggest that the gonadotrophin is perhaps more of a follicle stimulating than an interstitial cell stimulating hormone. On the other hand, follicular maturation in the ovaries is almost invariably followed with hemorrhage and marked luteinization and this suggests secretion of some ICSH. Leydig cells in males bearing transplantable thyrotrophic tumors appear highly active (5).

Bile ducts were enlarged more frequently in the females. The significantly lower frequency of large bile ducts in strain 101D and the absence in controls suggests that something in the tumor is responsible for the hypertrophy. This remarkable cystic dilatation of the extrahepatic biliary duct has been discussed elsewhere (6). A similar cystic dilatation of extrahepatic biliary duct has not been seen in large numbers of adrenototrophic and mammotropic tumors nor have they been seen in androgenic and estrogenic transplantable ovarian tumors and hormone-secreting tumors of the adrenal. Thus, they are characteristic for thyrotrophic pituitary tumors, yet may have some relation to gonadal hormones, since they were noted (7) in mice that were chronically treated with estrogens.

Concentration of TSH in Tumors: The potency of tumor powder will vary with the care in collecting and preparing the dry powder as well as

with the original concentration of TSH. The pituitary glands of normal mice contain 0.1–0.2 units of TSH per mg. dry weight, or about 0.04–0.08 units per pituitary (Table 3). Enlarged pituitaries (20–90 mg.) about 1 year after radiothyroidectomy have a normal concentration of TSH, i.e., 0.1–0.2 units per mg. dry weight. All of the tumor strains tested have had approximately this concentration of TSH in the dry tumor powder in early generations. For a 5 gm. tumor this represents about 100 units per tumor, or about 1,000–2,000 times the amount in the pituitary gland. Strain 77D has maintained its potency through the 5 generations followed in our laboratory. Strain 447D has shown a progressive decrease in potency with

TABLE 3. VARIATION OF CONCENTRATION OF TSH IN TUMORS AND BLOOD OF MICE FROM SUCCESSIVE GENERATIONS OF THREE STRAINS OF TUMOR

Generation	Tumor powder			Blood serum		
	77D	101D	447D	77D	101D	447D
	U/mg.	U/mg.	U/mg.	U/ml.	U/ml.	U/ml.
0	0.1	0.08	0.17			1.5
1	0.14	0.07	0.04			1.0
2A	0.15	0.12	0.023			0.75
2B	0.09	0.05	0.02	1.2	1.0	0.75
3A	0.12	0.05	0.01	1.1	0.5	0.6
3B	0.04	0.04		0.8	0.7	
4A	0.13	0.07		0.8	0.6	

TSH levels in normal mouse { Blood serum = 0.0005 U/ml. / Pituitary powder = 0.1–0.2 U/mg.

each generation, such that after 3 generations the potency is only one-tenth of the initial potency. With this decrease in potency a decrease in vascularity of the tumors occurred. Since the late "B" tumor transfers tend to be lower in potency than the early transfers, the desirability of early transfer is suggested.

Concentration of TSH in Blood Serum: Blood has been routinely collected from the mice only during the last two years. During this time there is an indication of decreasing potency with each transfer (Table 3). The TSH concentration of the serum does not appear to closely reflect that in the tumor because serum from generation 3A tumors (447D vs. 77D) differ in potency by a factor of about 2, while the tumor powders differ by a factor of about 10.

Blood serum from normal mice contains about 0.0005 units per ml. of TSH. Blood serum from mice bearing TSH tumors is 1,000–2,000 times more potent. The magnitude of this difference is approximately that between the TSH content of one mouse pituitary weighing 2 mg and a tumor weighing 5 gm.

Two isolated observations have been made:—One tumor from generation 2B-strain 447D contained a cyst filled with about 5 ml. of clear fluid. This fluid contained 0.45 units per ml. of TSH, which is approximately the

concentration in blood serum. Bile, collected from a mouse with an enlarged bile duct, was found to contain 5 units of TSH, per ml. a concentration which is higher than ever found for blood serum. This may not be a true value due to the presence of other constituents of the bile, but clearly demonstrates a high concentration of TSH in the bile. This TSH is probably destroyed in the gut and the bile is thus a significant route for the elimination of protein hormones from the body.

SUMMARY

The thyrotrophin potency of three stains of transplantable pituitary tumors has been followed through 4 or more transfer generations in athyroid mice. In all three stains the concentration of TSH in the tumors in early generations was about that in the normal mouse pituitary (0.1–0.2 U.S.P. units per mg. dry weight). In two strains the TSH concentration did not change, but in the third tumor strain the TSH concentration fell gradually to one-tenth of the original potency.

The concentration of TSH in the blood serum of mice with large tumors was about 1 unit per ml or 2,000 times the normal level and it decreased slightly with each transfer generation.

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THE UPTAKE AND METABOLISM OF S^{35} THIOUREA AND THIOURACIL BY THE THYROID AND OTHER TISSUES[1,2]

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THE anti-thyroid drugs have been utilized in the study of the metabolism of iodide by the thyroid and in the treatment of thyroid disease since 1943, yet the precise mode and site of action of these drugs remain unknown. The mechanism for the formation of protein-bound iodine in the thyroid is unknown. It is current biochemical practice in evaluating a metabolic process to study the action of an inhibitor on this metabolic process. Since the anti-thyroid drugs inhibit the formation of protein-bound iodine in the thyroid, knowledge concerning their mode and site of action may help in defining the mechanism involved in the iodination process in the thyroid. For a review of the literature on the anti-thyroid drugs, the reader is referred to several comprehensive articles (1, 2, 3, 4).

It is the purpose of this study to determine: 1) the effect of a single dose of thiourea and thiouracil on the formation of protein-bound iodine (PBI^{131}) in the thyroid of the rat; 2) the uptake and metabolism of S^{35} thiourea and S^{35} thiouracil by the thyroid and other tissues of the rat, and 3) the uptake and metabolism of S^{35} thiourea and S^{35} thiouracil by thyroid slices and thyroid cell particulate fractions.

METHODS

I. *Animals*

(a) *Determination of PBI^{131} in the thyroid.* Equimolar doses of thiourea and thiouracil were each administered intraperitoneally as a single dose to each of two groups of male rats weighing 150–200 gm. A dose of fifty μc. of I^{131} was then given intraperitoneally at intervals over a 24-hour period following the administration of the anti-thyroid drugs. The I^{131} was given to several control rats throughout the course of the experiment. The animals were killed by ether and exsanguination 15 minutes following the I^{131} injection. The thyroids were quickly removed, weighed, and the uptake of I^{131} determined as per cent of the administered dose. Each thyroid was homogenized in a chilled Potter-

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[2] Read before the American Goiter Association at the 1957 meeting held in New York, N. Y.

Elvehjem homogenizer with 0.5 ml. distilled water. Thirty microliter aliquots of the homogenate were applied along a 2.0 cm. line to Whatman #1 chromatography paper. Duplicate chromatograms were run in an ascending system of n-butanol-dioxane-ammonia (40:10:50) (v:v) and n-butanol-acetic acid-water (40:10:50) (v:v) for 18 hours. Radioactive spots were determined by the application of No-Screen x-ray film to the chromatogram. There were two radioactive spots, one at the origin which was assumed to be PBI^{131} and an iodide (I^{131}) spot determined by the use of potassium iodide (I^{127}) as a carrier which was stained with palladium chloride. In quantitating the amount of radioactivity at each of these two spots as a per cent of the total radioactivity along the chromatogram, the paper was cut into small uniform areas and counted in a well-type scintillation counter (Sensitivity: one μc. $I^{131} = 400{,}000$ NCPM).

(b) S^{35} *thiourea and* S^{35} *thiouracil administered to rats.* The S^{35} thiourea and S^{35} thiouracil were obtained from the Abbott Laboratories (Chicago, Illinois) and the New England Nuclear Corporation (Boston, Massachusetts). The specific activity ranged from 3.1 to 7.2 millicuries per millimole (mc./mM.). The S^{35} thiourea was easily dissolved in distilled water, the S^{35} thiouracil in distilled water plus dilute NaOH (final pH 7.5). These drugs were stored in the deep freeze, since they decompose if kept at room temperature. The purity of these samples was checked by chromatography using a butanol-HOH solvent (86:14) (v:v). A spot whose Rf was similar to that of standard thiourea and thiouracil solutions accounted for 99.0% of all the radioactivity on the chromatogram.

Male Sprague-Dawley rats weighing 150–175 gm. were maintained on Purina Fox Chow Pellets[3] and water *ad libitum*. The S^{35} thiourea and S^{35} thiouracil were administered intraperitoneally to rats with presumed normal thyroid function. At intervals over a 24-hour period the animals were killed with ether and exsanguinated. The thyroid was removed, cleaned, weighed, and then homogenized in 0.5 ml. distilled water. A piece of liver, 150–200 mg., was removed, weighed, and homogenized in distilled water (100 mg. of tissue per 1.0 ml.). The blood was centrifuged and the serum removed. Appropriate aliquots of the homogenates, the serum, and S^{35} standards were dried on paper disks in a constant area 1.0 cm. in diameter and the radioactivity assayed by a thin end window Geiger-Mueller tube (window thickness: 1.0 mg./cm.^{2}). The background of this counting apparatus was 20 counts per minute; the efficiency, assayed by counting a series of beta ray emitter standards, was 3.5% for S^{35}. All samples were counted in duplicate, each for a total of 4096 counts. Control studies revealed that the use of small uniform aliquots of the homogenates (0.50 mg. protein or less) obviated the need for a self-absorption correction factor in assaying the S^{35} radioactivity.

Aliquots of the thyroid and liver homogenates, the serum and appropriate S^{35} standards were also placed on Whatman #1 paper for chromatographic analysis. Duplicate chromatograms were placed in butanol-HOH (86:14) (v:v) and ethanol-ammonium acetate[4] (1.0 M) pH 7.5 (7.5:3) (v:v) for 18 hours, removed, and dried. The concentration of radioactivity along the paper was assayed by closely approximating No-Screen x-ray film to the paper for variable periods, and then by cutting up the paper, placing the cut sections on planchettes, and counting the S^{35} radioactivity as noted above. Control studies revealed that 90 to 95% of the S^{35} radioactivity applied to the chromatograms was recovered.

II. *Thyroid Slices*

Thyroids were obtained from sheep, steer, and pigs that were killed at local abattoirs. They were then wrapped in cellophane, placed on ice, brought to the laboratory, im-

[3] Total I^{127} content 3.0 micrograms/gm. of food.

[4] Leloir's solvent (5).

mediately cleaned of extraneous tissue, and sliced with a razor by hand. Prior to use, the slices were immersed in chilled isotonic sodium chloride. Representative slices were weighed on a torsion balance and analyzed for their iodine (I^{127}) content[5] prior to and following their immersion in chilled sodium chloride. The I^{127} content and functional activity of the thyroid tissue varied so widely that, whenever possible, a complete experiment was carried out on one thyroid gland. Part of the same thyroid was used to prepare particulate fractions, and part was placed in the deep freeze to produce frozen tissue for comparative observations with the fresh slice and fresh particulate experiments.

Fresh sheep thyroid slices weighing about 200 mg. were included in standard 25-ml. Erlenmeyer flasks with Krebs Ringer Bicarbonate buffer (KRB) and various concentrations of S^{35} thiourea and S^{35} thiouracil. The final volume of the incubation media was 3.0 ml., and the final pH was 7.4. The flasks were tightly stoppered and were shaken in a water bath at a temperature of 38° C. At intervals, the slices were removed from the media, pressed gently against the side of the flask, and homogenized in chilled glass Potter-Elvehjem homogenizers with 1.0 ml. of distilled water per 100 mg. of tissue. In preparing rat thyroid slices, the two lobes of a thyroid (20 ± 5 mg.) were sliced in half with a razor and the entire thyroid was placed in a 7.0-ml. Warburg vessel. The final volume of the incubation medium was 1.0 ml. These slices were homogenized in 0.5 ml. of water. Aliquots of the chilled homogenates, the respective media, and appropriate S^{35} standards were assayed for S^{35} radioactivity by counting methods described above and for the nature of the S^{35} fraction by paper chromatography.

Thyroid homogenates (20%) were precipitated with perchloric acid (0.6 N) and the acid soluble fraction, phospholipid fraction, the ribose nucleic acid (RNA) and the desoxyribosenucleic acid (DNA) fractions were separated (7). Aliquots of each fraction were then counted for S^{35} radioactivity and were analyzed chromatographically.

The following procedure was carried out to separate the cellular particulate fractions of sheep and steer thyroids. The thyroids were homogenized in a chilled Waring Blendor for 30 seconds with 2 volumes of 0.25 *M* sucrose-0.08 *M* $KHCO_3$. The homogenate was then filtered through two layers of cheese cloth, and the various cellular fractions were separated by differential centrifugation. The homogenate was spun twice for 5 minutes in a refrigerated International centrifuge at $600 \times g$. The supernatants were decanted and combined (Supernatant I). The precipitates were combined and constitute the nuclear fraction and cellular debris. Supernatant I was spun for 15 minutes at $9000 \times g$ to remove the mitochondria. The resultant supernatant (II) was then spun for 60 minutes at $105{,}000 \times g$ in a Spinco Preparative Centrifuge to remove the microsomes. Occasionally, supernatant I was spun directly for 60 minutes at $105{,}000 \times g$ which removed mitochondria and microsomes together. These particulate fractions were diluted with Krebs Ringer Bicarbonate so that 1.0 ml. represented 1.5 to 2.0 gm. of the original thyroid tissue.

RESULTS

Animal Experiments

I. *Anti-thyroid drugs and inhibition of PBI^{131} in the thyroid*

The concentration of these drugs in the thyroid necessary to inhibit completely the formation of PBI in the thyroid is unknown. The following observations were made in an attempt to clarify this problem. Since the chronic administration of these drugs produces uncontrollable variables in thyroid physiology, the inhibition of the formation of PBI^{131} by the thyroid

[5] Performed by a modification of the Chaney Method (6).

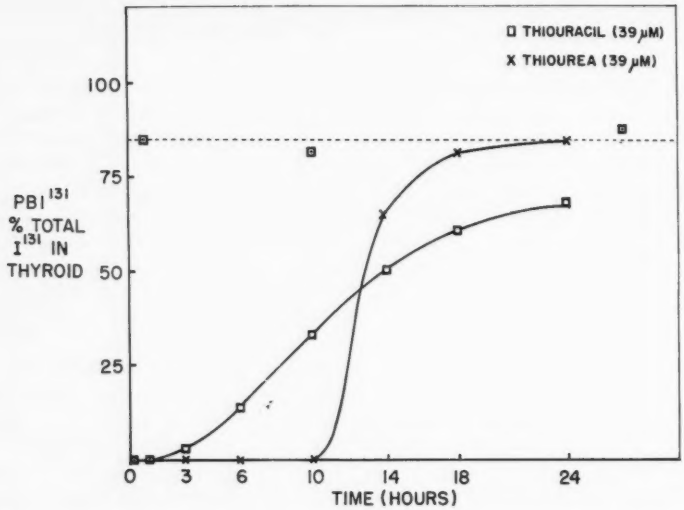

FIG. 1. The effect of the administration of a single equimolar dose of thiourea (39 μM.) and thiouracil (39 μM.) on the formation of PBI^{131} in the thyroid of the rat.
⊡ Control animals sacrificed throughout the course of the experiment.

has been studied following the administration of a single dose of thiourea and thiouracil.

Equimolar doses of thiourea (39 micromoles; 3.0 mg.) and thiouracil (39 micromoles; 5.0 mg.) were each administered as a single dose intraperitoneally to two groups of twelve male rats. This single dose of thiourea completely blocks the formation of PBI^{131} in the thyroid for at least 10 hours; at the 14-hour interval the per cent of PBI^{131} increases rapidly to 64% (Fig. 1). This inhibition of PBI^{131} formation in the thyroid by thiourea is to be contrasted with that by thiouracil. In the animals which had received thiouracil the block ceases to be complete at the 6-hour interval and then gradually decreases over the 24-hour period. Unlike thiourea, the inhibition by thiouracil is still evident at the 24-hour interval.

II. *Uptake and metabolism of S^{35} thiourea and S^{35} thiouracil by rat thyroid*

In an attempt to evaluate the factors involved in Experiment I, S^{35} thiourea and S^{35} thiouracil were given intraperitoneally to rats. The uptake of the S^{35} radioactivity by the thyroid gland was followed over a 24-hour interval and compared with the level of S^{35} radioactivity found in the liver and serum. The nature of the S^{35} radioactivity in the thyroid, liver, and serum was determined by chromatographic study.

S^{35} thiourea: Fifteen minutes after the intraperitoneal administration of 39 micromoles (3.0 mg.) of S^{35} thiourea[6] to rats, there is 0.72% of the ad-

[6] Specific activity: 7.2 millicuries/millimole.

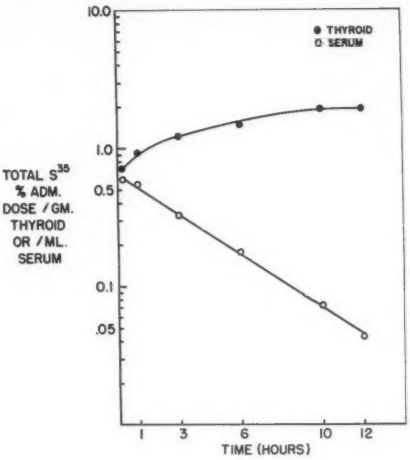

FIG. 2. Semilogarithmic plot of the per cent of the administered dose of S^{35} radioactivity per gram of thyroid and per ml. of serum following the administration of a single dose of 39 μM. (3.0 mg.) of S^{35} thiourea to normal rats. Data at each time interval are derived from one animal.

ministered dose of S^{35} radioactivity per gram of thyroid (Fig. 2). Ten hours after the injection, the maximum uptake in the thyroid is reached at 1.95% of the administered dose. At the 15-minute interval, the level of total S^{35} radioactivity per ml. of serum is equal to that per gram of thyroid. Thereafter, the S^{35} radioactivity in the serum decreases exponentially with a half-life of about 3 hours. In 10 hours, the level of total S^{35} radioactivity per gram of thyroid is 40 times that per ml. of serum. These data suggested a specific concentration mechanism in the thyroid for S^{35} thiourea.

Chromatographic analysis of the S^{35} radioactivity in the thyroid and serum is noted in Table 1. In 10 hours only 3.4% of the total S^{35} radioactivity in the thyroid travels with the same Rf as S^{35} thiourea. This is to be contrasted with the serum in which 75.0% of the total S^{35} radioactivity travels with the same Rf as S^{35} thiourea. Hence, only a small fraction of the

TABLE 1. CHROMATOGRAPHIC ANALYSIS OF THE S^{35} RADIOACTIVITY AND DETERMINATION OF THE LEVEL OF S^{35} THIOUREA IN THE THYROID AND SERUM OF NORMAL RATS GIVEN 39 MICROMOLES (3.0 MG.) OF S^{35} THIOUREA (S*T) INTRAPERITONEALLY

	Time rat killed after adm. of S*T	S^{35} spots, % of total S^{35} along the chromatogram†					Total S^{35} per gm. thyroid, per ml. serum		Actual S*T per gm. thyroid and per ml. serum (micromoles)‡
		R_f 0.0 origin	0.24 S^{35} sulfate	0.60 x	0.74 S^{35} thiourea	0.86 y	% adm. dose	Micromol. S*T	
Thyroid	15 min.	3.7	8.7	5.5	74.8	2.5	0.72	0.28	0.21
	1 hour	6.4	22.0	14.4	44.5	6.4	0.95	0.37	0.16
	3 hours	13.5	34.0	18.4	31.5	—	1.20	0.47	0.15
	6 hours	16.3	52.6	16.9	6.1	1.2	1.51	0.59	0.036
	10 hours	13.4	56.0	18.0	3.4	—	1.95	0.76	0.026
	12 hours	11.6	80.1	2.8	2.9	—	1.79	0.70	0.020
	14 hours	12.1	79.4	5.1	—	—	1.82	0.71	—
Serum	15 min.	2.5	2.5		92.5		0.59	0.23	0.21
	1 hour	2.7	3.4		88.5		0.57	0.22	0.19
	6 hours	5.2	12.1		80.2		0.18	0.07	0.056
	10 hours	6.0	15.0		75.0		0.08	0.03	0.022

† Solvent system, Leloir's; S^{35} spots x and y are unknown.
‡ This corrected value obtained by multiplying total S^{35} in thyroid and serum by % S^{35} Thiourea obtained from chromatogram.

total S^{35} radioactivity in the thyroid is S^{35} thiourea. Determination of the actual amount of S^{35} thiourea in the thyroid and serum reveals that the concentration of S^{35} thiourea per gram of thyroid and per ml. of serum is equal. Hence, there is no concentration gradient for S^{35} thiourea between the thyroid and serum. Thyroid to serum concentration gradient for total S^{35} radioactivity is explicable by the metabolism of S^{35} thiourea by the thyroid. Further analysis of the chromatographic S^{35} fractions in the thyroid reveals that at 10 hours, 56.0% of the total S^{35} radioactivity moves with the same Rf as S^{35} sulfate,[7] and 13.4% remains at the origin of the chromatogram, presumably bound to protein. This is to be contrasted with the serum in which 15.0% travels as S^{35} sulfate and 6.0% remains at the origin. There are two unidentified spots on the chromatogram of the thyroid; no comparative S^{35} spots are present on the chromatogram of the serum.

The thyroid of the rat has an effective mechanism for metabolizing S^{35} thiourea (Table 1). In 15 minutes following the intraperitoneal administration of 39 micromoles (μM) of S^{35} thiourea to rats, there is 0.72% of the administered dose of S^{35} radioactivity per gram of thyroid. Assuming this S^{35} to be S^{35} thiourea, there is 39 $\mu M \times 0.0072$ or 0.28 μM of S^{35} thiourea per gram of thyroid. However, chromatographic analysis reveals that only 74.8% of the total S^{35} radioactivity in the thyroid is S^{35} thiourea; hence, 0.28 $\mu M \times 0.252$ or 0.071 μM of S^{35} thiourea were metabolized in 15 minutes. In 10 hours, there is 1.95%[8] of the administered dose of S^{35} radioactivity per gram of thyroid. This represents a tissue level of 39 $\mu M \times 0.0195$ or 0.76 μM of S^{35} thiourea per gram of thyroid. However, only 3.6% of this is S^{35} thiourea; hence, 0.76 $\mu M \times 0.964$ or 0.734 μM were metabolized. This leaves a concentration of S^{35} thiourea in the thyroid of 0.760 minus 0.734 or 0.026 micromoles/gram (2.6×10^{-5} M) ten hours following the administration of 39 micromoles of S^{35} thiourea to rats.

The S^{35} thiourea experiment was repeated using half the dose of S^{35} thiourea. Fifteen minutes after the intraperitoneal administration of 20 micromoles of S^{35} thiourea,[9] 0.75% of the administered S^{35} radioactivity is found per gram of thyroid; in 5 to 6 hours the maximum uptake has been reached at a level of 2.1% of the administered dose (Table 2). There is a marked difference between the metabolism of the S^{35} thiourea by the thyroid and by an equal weight of liver. In 6 hours, 6% of the total S^{35} radioactivity in the thyroid moves with the same Rf as S^{35} thiourea. This is to be

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[7] Sodium S^{35} sulfate used as the control. The nature of this spot was ascertained by running the chromatogram in Leloir's solvent in which the Rf of S^{35} sulfate is 0.24 and in butanol-HOH solvent in which S^{35} sulfate remains at the origin. Its nature was further verified by paper electrophoresis using Whatman #30 paper, ammonium acetate buffer, 0.2 M, pH 3.8, 1000 volts, 6–8 milliamp. for 35 minutes.

[8] A minimum figure, assumes that no S^{35} radioactivity has left the thyroid in this interval.

[9] Specific activity: 7.2 millicuries/millimole.

TABLE 2. CHROMATOGRAPHIC ANALYSIS OF THE S^{35} RADIOACTIVITY AND DETERMINATION OF THE LEVEL OF S^{35} THIOUREA IN THE THYROID AND SERUM OF NORMAL RATS GIVEN 20.0 MICROMOLES (1.52 MG.) OF S^{35} THIOUREA (S*T) INTRAPERITONEALLY

	Time rat killed after adm. of S*T	S^{35} spots, % of total S^{35} along the chromatogram†					Total S^{35} per gm. thyroid, liver; per ml. serum		Actual S*T per gm. thyroid, liver; per ml. serum (micromoles)
		R_f 0.0 origin	0.24 S^{35} sulfate	0.60 x	0.74 S^{35} thiourea	0.86 y	% adm. dose	Micromol. S*T	
Thyroid	15 min.	4.7	24.0	10.9	55.2	1.9	0.75	0.15	0.081
	1 hour	8.9	37.2	17.0	29.1	2.8	1.10	0.22	0.065
	3 hours	16.4	43.5	16.5	14.5	4.1	1.70	0.34	0.049
	5 hours	10.5	69.5	7.0	8.0	1.2	2.1	0.42	0.034
	6 hours	11.4	69.5	6.2	6.4	—	2.0	0.41	0.026
Serum(S) and Liver(L)	1 hour(S)	3.0	10.2	—	86.0	—	0.42	0.084	0.072
	(L)	2.6	2.3	—	91.0	—	0.30	0.060	0.055
	3 hours(S)	3.4	8.8	—	82.5	—	0.24	0.047	0.039
	(L)	2.7	4.6	—	83.5	—	0.24	0.047	0.039
	6 hours(S)	4.0	14.0	—	80.1	—	0.11	0.022	0.018
	(L)	2.7	10.4	6.0	75.0	—	0.12	0.024	0.018

† Solvent system, Leloir's; S^{35} spots x and y are unknown.

contrasted with the liver in which 75% of the S^{35} radioactivity in 6 hours behaves like S^{35} thiourea (Fig. 3).

Five hours following the intraperitoneal administration of 20 μM of S^{35} thiourea to rats, 0.386 μM were metabolized per gram of thyroid. Following the administration of 40 μM of S^{35} thiourea to rats, 0.734 μM were metabolized per gram of thyroid, but not for 10 hours. Apparently the capacity of the thyroid to metabolize S^{35} thiourea is saturated by the administration of 20 μM of S^{35} thiourea.

Sodium S^{35} sulfate: Ten micromoles of sodium sulfate[10] labeled with S^{35} were administered to rats for comparison with the experiments with S^{35} thiourea.

It is clear from Fig. 4 that the magnitude and pattern of concentration of $NaS^{35}O_4$ differs from that of S^{35} thiourea by the rat thyroid. Chromatographic analysis of the S^{35} radioactivity in the thyroid reveals that the majority of the S^{35} radioactivity travels as S^{35} sulfate, and some remains at the origin presumably bound to protein.

S^{35} thiouracil: Studies with S^{35} thiouracil were carried out similar to those for S^{35} thiourea. A single intraperitoneal injection of 39 micromoles (5.0 milligrams) of S^{35} thiouracil[11] was given to each of six male rats.

The maximum level of total S^{35} radioactivity per gram of thyroid occurred in 15 to 30 minutes and then decreased exponentially with a half-life of about 4.8 hours (Fig. 5). This curve of total S^{35} radioactivity in the thyroid is obviously different from that of rats given an equimolar dose of S^{35} thiourea (Fig. 2). It is also obvious that there is no concentration gradient for the S^{35} radioactivity between the thyroid and the serum.

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[10] Specific activity: 20 millicuries/millimole.

[11] Specific activity: 3.1 millicuries/millimole.

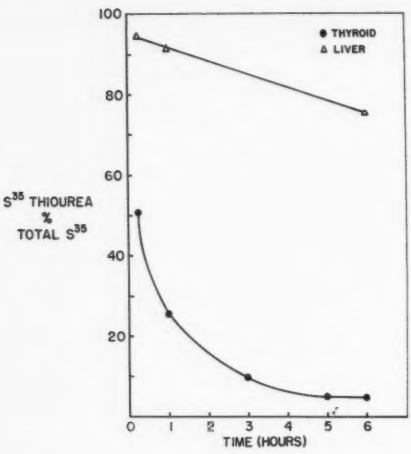

FIG. 3. S^{35} thiourea as per cent of total S^{35} radioactivity per equal weight of thyroid and liver following the administration of 20 μM. (1.5 mg.) of S^{35} thiourea to normal rats.

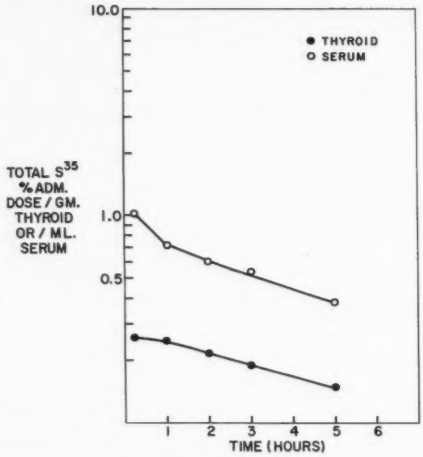

FIG. 4. Semilogarithmic plot of the per cent of S^{35} radioactivity per gram of thyroid and per ml. of serum following the administration of a single dose of 10 μM. of sodium S^{35} sulfate to normal rats. Data at each time interval are derived from one animal.

It would appear from comparing Fig. 2 and Fig. 4 that the metabolism of S^{35} thiouracil by the thyroid of the rat is different from that of S^{35} thiourea. Chromatographic analysis of the S^{35} radioactivity in the thyroid reveals this to be true (Table 3). The S^{35} thiouracil is converted in part to S^{35} sulfate, but largely to several other S^{35} fractions which have not been identified. One is able to calculate that a concentration of 0.023 μM of S^{35} thiouracil is present per gram of thyroid 6 hours after the administration of 39 μM of S^{35} thiouracil to rats.

Attempts to alter metabolism of S^{35} thiourea in rat thyroid

Several experiments have been carried out in an attempt to alter the metabolism of S^{35} thiourea by the thyroid.

a. *Effect of hypophysectomy.* A single dose of 20 μM of S^{35} thiourea was

TABLE 3. CHROMATOGRAPHIC ANALYSIS OF S^{35} RADIOACTIVITY AND DETERMINATION OF THE LEVEL OF S^{35} THIOURACIL IN THE THYROID OF NORMAL RATS GIVEN 39 MICROMOLES (5.0 MG.) OF S^{35} THIOURACIL (S*TU) INTRAPERITONEALLY

	Time rat killed after adm. of S*TU	S^{35} spots, % of total S^{35} along the chromatogram†					Total S^{35} per gm. thyroid		Actual S*TU per gm. thyroid (micromoles)
		R_f 0.0 Origin	0.25 S^{35} sulfate	0.59 A	0.76 S^{35} thiouracil	0.90 B	% adm. dose	Micromol. S*TU	
Thyroid	15 min.	3.2	2.4	7.6	75.0	1.2	0.52	0.20	0.15
	6 hours	12.2	14.3	26.5	26.6	12.2	0.22	0.086	0.023

† Solvent system, Leloir's; S^{35} spots A and B are unknown.

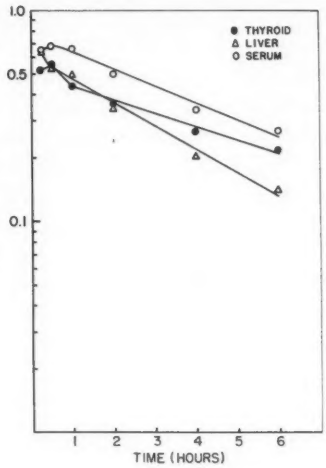

FIG. 5. Semilogarithmic plot of the per cent of the administered dose of S^{35} radioactivity per gram of thyroid, liver, and per ml. of serum following the administration of a single dose of 39 μM. (5.0 mg.) of S^{35} thiouracil to normal rats. Data at each time interval are derived from one animal.

given intraperitoneally to male rats weighing 125–150 gm. that had been hypophysectomized 6 days prior to this experiment. Fifteen minutes following the injection of S^{35} thiourea, the uptake of S^{35} radioactivity per gram of thyroid is 0.53% of the administered dose (Table 4). This value is not impressively different from the comparative one of 0.75% for the normal rats (Table 2). The subsequent course of events is different from that for the normal rats in that the uptake of S^{35} radioactivity per gram of thyroid remains fairly constant over a 5-hour period.

Chromatographic analysis of the S^{35} radioactivity in the thyroid and serum is found in Table 4. Sixty-nine per cent of the S^{35} radioactivity in the thyroid is S^{35} thiourea in 5 hours. This is to be contrasted with the figure of 8.0% for the normal rats. Furthermore, the unidentified S^{35} spots (x and

TABLE 4. CHROMATOGRAPHIC ANALYSIS OF S^{35} RADIOACTIVITY AND DETERMINATION OF THE LEVEL OF S^{35} THIOUREA IN THE THYROID AND SERUM OF HYPOPHYSECTOMIZED RATS GIVEN 20 MICROMOLES OF S^{35} THIOUREA (S*T) INTRAPERITONEALLY

	Time rat killed after adm. of S*T	S^{35} spots, % of total S^{35} along the chromatogram†			Total S^{35} per gm. thyroid, per ml. serum		Actual S*T per gm. thyroid and per ml. serum (micromoles)
		Rf 0.0 Origin	0.24 S^{35} sulfate	0.74 S^{35} thiourea	% adm. dose	Micromol. S*T	
Thyroid	15 min.	1.9	—	84.2	0.53	0.106	0.089
	1 hour	1.7	—	86.4	0.63	0.126	0.109
	2 hours	3.7	6.2	80.8	0.61	0.122	0.099
	3 hours	4.1	6.2	82.5	0.53	0.106	0.088
	4 hours	5.1	6.4	80.7	0.56	0.111	0.090
	5 hours	7.5	14.9	68.7	0.51	0.102	0.070
Serum	4 hours	1.7	10.9	84.9	0.66	0.132	0.112
	5 hours	2.3	16.4	78.4	0.53	0.106	0.083

† Solvent System, Leloir's.

y) found in the chromatogram of normal rat thyroids are not found here. This experiment was repeated on two other occasions, and the results were similar.

b. *Effect of potassium iodide (KI).* Five hundred micrograms of KI were given intraperitoneally to four normal rats one hour prior to the intraperitoneal administration of 20 micromoles (1.5 mg.) of S^{35} thiourea. Control animals were given an injection of water equal in volume to that used for the KI injection; then one hour later, 20 micromoles of S^{35} thiourea. At the one and 6-hour intervals, the animals that had been given KI take up 29.4 and 33.0% less of the administered dose of S^{35} per gram of thyroid than the control animals (Table 5). This is due to a decrease in the metabolism of

TABLE 5. THE EFFECT OF A SINGLE DOSE OF 500 MICROGRAMS OF POTASSIUM IODIDE (KI) ON THE METABOLISM OF S^{35} THIOUREA (S*T) BY THE THYROID OF THE NORMAL RAT

	Time rat killed after adm. of S*T (hours)	S^{35} spots, % of total S^{35} along the chromatogram†					Total S^{35} per gm. of thyroid		Actual S*T per gm. thyroid (micromol.)	S*T metab. per gm. thyroid (micromol.)
		R_f 0.0 origin	0.25 S^{35} sulfate	0.60 x	0.74 S^{35} Thiourea	0.86 y	% adm. dose	Micromol. S*T		
Control	1	8.9	37.2	17.0	29.1	2.5	1.12	0.224	0.065	0.159
KI		13.5	24.0	18.5	37.0	5.0	0.79	0.158	0.058	0.100
Control	6	11.4	69.5	6.2	8.9		2.00	0.400	0.030	0.370
KI		19.0	46.6	20.1	9.5		1.34	0.268	0.025	0.243
Control	10	12.8	73.7	6.6	3.3		1.47	0.294	0.009	0.285
KI		17.9	49.0	16.9	11.3		1.00	0.200	0.022	0.178
Control	14	16.0	72.3	5.4	1.8		0.51	0.102	0.002	0.100
KI		14.1	74.6	5.0	1.6		0.90	0.180	0.003	0.177

† Solvent system, Leloir's; S^{35} spots, x and y are unknown.

S^{35} thiourea by the thyroid of rats given KI; 37.4% less at the one-hour interval and 33.4% at the 6-hour interval in comparison to the control animals. The chromatographic data suggest that the effect of KI persists for at least 10 hours and is over in 14 hours. The results obtained from two other control experiments would suggest that this effect of KI on the metabolism of S^{35} thiourea is significant.

III. *Incubation of thyroid slices with S^{35} thiourea and S^{35} thiouracil*

The uptake of S^{35} thiourea (10^{-3} *M*) by sheep thyroid and liver slices (200 mg.) is noted in Fig. 6. An analysis of the S^{35} radioactivity in the slices 0, 15, 30, 45, 60, and 120 minutes from the time of incubation reveals the peak level of the radioactivity at 15 to 30 minutes. Thereafter, the uptake curve is level with a slight dropping off at the 120-minute interval. Chromatographic analysis of the S^{35} radioactivity in the thyroid and liver slices reveals it to be S^{35} thiourea. Hence, the S^{35} thiourea is not metabolized by the thyroid or liver slices during the period of incubation. Incubating the fresh thyroid slices with S^{35} thiourea 10^{-5} *M*, 2 and 4×10^{-5} *M*, 10^{-4} *M*, 2, 4, 6, and 8×10^{-3} *M* produces levels in the tissue commensurate with

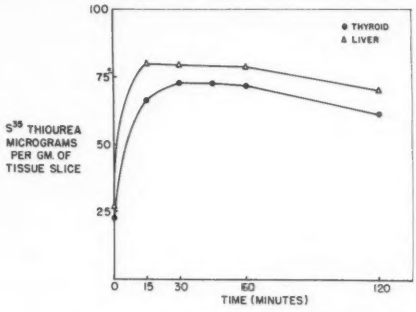

FIG. 6. The uptake of S^{35} thiourea by sheep thyroid (200 mg.) and sheep liver slices (200 mg.) after incubation of the slices with S^{35} thiourea (10^{-3} *M*) in 3.0 ml. of Krebs Ringer Bicarbonate buffer (KRB).

* Refers to level at which the concentration of S^{35} thiourea in the tissue slice $= 10^{-3}$ *M*.

those in an equal weight of medium. Hence, there is no concentration gradient for S^{35} thiourea in tissue versus medium. The pattern and magnitude of uptake for S^{35} thiourea by frozen and boiled sheep thyroid slices, fresh rat thyroid slices, and fresh sheep and rat liver slices is similar to that by the fresh sheep thyroid slices.

Iodides, as potassium iodide, in concentrations varying from 10^{-2} *M* to 10^{-5} *M* did not affect the uptake of S^{35} thiourea by sheep thyroid slices (Table 6).

S^{35} thiouracil: The uptake curve of S^{35} radioactivity by fresh sheep thyroid slices incubated with S^{35} thiouracil (10^{-3} *M*) in KRB is similar to that for S^{35} thiourea (Fig. 7). Other similarities consist of: 1) no alteration of the S^{35} thiouracil by the tissue; 2) no concentration gradient for the S^{35} thiouracil in the thyroid slices over the medium, and 3) concentrations of KI ranging from 10^{-2} *M* to 10^{-5} *M* in the incubation medium do not affect the level of S^{35} thiouracil in the thyroid slices.

Attempts were made to displace the S^{35} thiouracil from the thyroid slices. Individual flasks containing thyroid slices (200 mg.) and S^{35} thiouracil (10^{-3} *M*) are incubated in Krebs Ringer Bicarbonate (KRB) at 38° C for 60 minutes. The thyroid slices are removed, pressed gently against the side of the flask, and are then reincubated at 38° C in separate flasks containing only KRB for 0, 15, and 60 minutes. Zero time consists of swirling

TABLE 6. THE EFFECT OF KI (I^{127}) ON THE UPTAKE OF S^{35} THIOUREA (S*T) BY SHEEP THYROID SLICES. INCUBATION FOR TWO HOURS IN 3.0 ML. Krebs Ringer Bicarbonate Buffer (KRB)

	Slice wt. (mg.)	I^{127},† molar conc. in medium	I^{127}, final conc. in slice (μg.)	S*T, molar conc. in medium	S*T, molar conc. in slice
1	200	—	375	1.0×10^{-3}M	1.3×10^{-3}M
2	203	—	312	1.0×10^{-3}M	1.2×10^{-3}M
3	200	1.0×10^{-5}M	400	1.0×10^{-3}M	1.4×10^{-3}M
4	208	1.0×10^{-4}M	394	1.0×10^{-3}M	1.3×10^{-3}M
5	204	1.0×10^{-3}M	680	1.0×10^{-3}M	1.3×10^{-3}M
6	210	1.0×10^{-2}M	1220	1.0×10^{-3}M	1.3×10^{-3}M

† Added to the KRB buffer and slices 5 minutes prior to the addition of S*T.

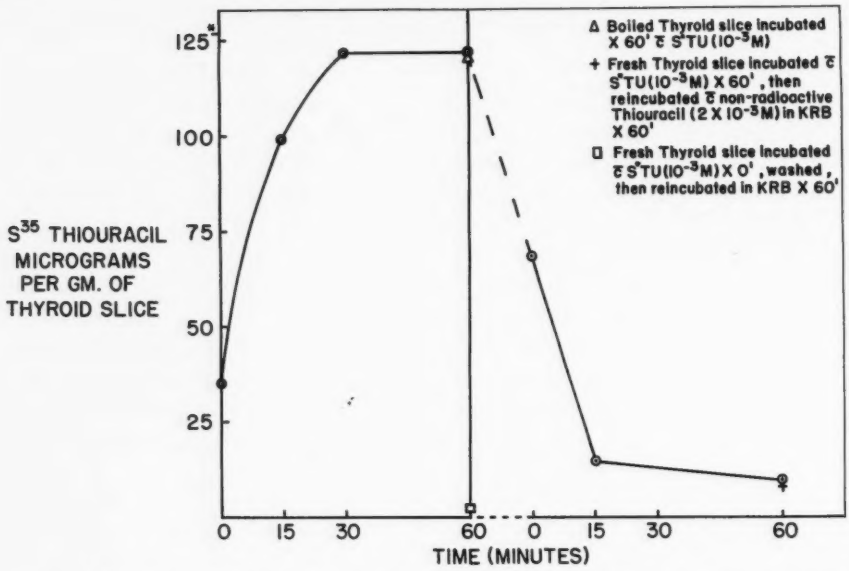

FIG. 7. The uptake (left) and release (right) of S^{35} thiouracil by sheep thyroid slices (200 mg.) after incubation of the slices with S^{35} thiouracil (10^{-3} *M*) in 3.0 ml. KRB.

* Refers to the level at which the concentration of S^{35} thiouracil in the tissue slice $= 10^{-3}$ *M*.

the slices six times in plain KRB. It can be seen from Fig. 7 that at 0 time about 33% of the S^{35} thiouracil is removed from the slices. Most of the S^{35} thiouracil has left the slices after re-incubation in plain KRB for 15 minutes. A small but insignificant fraction of the S^{35} radioactivity remains in the slices even after re-incubation for 60 minutes. This level of S^{35} thiouracil in the slices is not effective in inhibiting the incorporation of I^{131} into PBI^{131} by the thyroid slices.

Fractionation of the thyroid slices into acid-insoluble, acid-soluble, phospholipid, RNA and DNA fractions indicates that all the significant S^{35} radioactivity is found in the acid soluble fraction. The washing out and the fractionation procedure with thyroid slices incubated with S^{35} thiourea reveal results similar to those obtained with S^{35} thiouracil.

Preliminary experiments have been carried out involving the incubation of mitochondria, microsomes, and the supernatant fraction of sheep thyroids with these S^{35} labeled drugs. Like the slice experiments, no metabolism of the S^{35} thiourea and S^{35} thiouracil was noted.

DISCUSSION

The observations made with S^{35} thiourea and S^{35} thiouracil reveal that there is a rapid accumulation of these drugs by the thyroid. Only a very

small fraction, 0.5 to 2.0%, of the administered dose is taken up by the thyroid. However, in 15 minutes following the intraperitoneal administration of thiourea and thiouracil to rats, the level of these drugs in the thyroid is sufficient to inhibit completely the formation of PBI^{131} in the thyroid. This is to be compared with the rapidity with which the thyroid synthesizes PBI^{131}. The formation of PBI^{131} in the thyroid has been reported to occur as early as 11 seconds following the administration of I^{131} to mice (8).

It has been stated that thiourea is an incompletely effective anti-thyroid substance in the rat (4). The present experiments demonstrate that the administration of a single dose of 39 μM. of thiourea completely blocked the formation of PBI^{131} in the thyroid of the rat for at least 10 hours. In animals which received an equimolar dose of thiouracil, the block ceased to be complete in 6 hours. Studies utilizing S^{35} thiourea and S^{35} thiouracil provide information which allows us to establish a level of these drugs in the thyroid necessary to inhibit the formation of PBI^{131} in the thyroid. A minimum level of 2.6×10^{-5} *M* of thiourea in the thyroid completely blocks the formation of PBI^{131} in the thyroid of the rat. A concentration of 2.3×10^{-5} *M* of thiouracil in the thyroid results in an 81% inhibition of the formation of PBI^{131} in the thyroid. Hence, it is suggested that with an equimolar concentration of thiourea and thiouracil in the thyroid, thiourea may be more effective than thiouracil in blocking the formation of PBI^{131} in the thyroid. One may speculate that the mechanism involved in the iodination process is more sensitive or more accessible to the action of thiourea than to the action of thiouracil.

Several comparisons may be made between the uptake of these S^{35} labeled anti-thyroid drugs and the uptake of iodide by the thyroid. There is no concentration gradient for S^{35} thiourea and S^{35} thiouracil between the thyroid and serum, and the presence of the pituitary is not an important factor in controlling the uptake of S^{35} thiourea by the thyroid. This is unlike iodide for which a definite concentration gradient exists between the thyroid and serum and for which the pituitary is important in regulating its uptake and concentration by the thyroid. It appears as though iodide does not directly affect the uptake of S^{35} thiourea by the thyroid. These data suggest that the uptake of thiourea is a passive process and is independent of the mechanism in the thyroid concerned with the concentration of iodide which is probably enzymatically controlled (9).

The *in vivo* experiments with rats demonstrate an interesting metabolism of these S^{35} labeled drugs by the thyroid of the rat. S^{35} thiourea is rapidly metabolized, and the labeled sulfur is chiefly converted to S^{35} sulfate. This is consistent with the observations of Shulman (10). S^{35} thiouracil is less rapidly metabolized, and the S^{35} is converted only in part to S^{35} sulfate in the same interval. Van Erkelens found that S^{35} 4-methyl thiouracil and S^{35} 4-carboxy thiouracil are not completely metabolized by the thyroid of

the cockerel in 24 hours (11). These observations plus the present experiment would explain the more prolonged inhibitory action of thiouracil versus thiourea on the formation of PBI^{131} in the thyroid. Until the unidentified breakdown products of S^{35} thiourea and S^{35} thiouracil are characterized, it is difficult to assign them a role in the antithyroid action.

The present data do not definitely establish whether the metabolism of thiourea per se is a requisite for its inhibitory effect on the iodination mechanism in the thyroid. If the metabolism is an essential factor in the action of these drugs, this would suggest that their mechanism of action is by competitive inhibition for the substrate involved in the oxidation of iodide to the active iodinating intermediate. The decrease in the metabolism of S^{35} thiourea in the thyroid of rats given KI is compatible with this hypothesis. Experiments in the hypophysectomized rat suggest that the metabolism is not essential. The metabolism of S^{35} thiourea is greatly reduced in the thyroid of the hypophysectomized rat, yet thiourea is as effective[12] in inhibiting the formation of PBI^{131} in the thyroid of this animal as in the thyroid of the normal rat.

The greatly decreased metabolism of S^{35} thiourea by the thyroid of hypophysectomized rats suggests that the thyroid stimulating hormone (TSH) and/or other pituitary hormones may be necessary for the metabolism of the S^{35} thiourea. The data of Taurog *et al.* (12) provide a parallel observation concerning the iodination mechanism in the thyroid. Their observations suggest that TSH is necessary for the formation of I^{131} labeled thyroxine by the thyroid of the hypophysectomized rat. Hence, a common denominator may be involved in the metabolism of S^{35} thiourea and the formation of I^{131} thyroxine by the thyroid. Additional evidence for this suggestion is as follows. Assuming that the thyroid of the rat produces 3.0 μg. of *l*-thyroxine per day, this calculates to 0.16 millimicromoles/hour. The thyroid (15 mg.) of a rat metabolized 1.10 millimicromoles of S^{35} thiourea per hour. These values are possibly of the same order of magnitude.

In view of the difference between the *in vivo* and the present *in vitro* experiments with S^{35} thiourea and S^{35} thiouracil, one must be cautious in transposing *in vitro* data obtained with these drugs to *in vivo* experiments.

The metabolism of S^{35} thiourea offers a tool other than I^{131} with which to study the iodination mechanism in the thyroid and the extra-thyroidal factor (or factors) which control this mechanism. This tool may be particularly valuable since the uptake of S^{35} thiourea by the thyroid is apparently not directly depressed by those factors which depress the uptake of I^{131} and which limit its usefulness.

SUMMARY

Observations have been made following the administration of S^{35}-labeled thiourea and thiouracil to rats and following the incubation of these

[12] Unpublished data.

compounds with thyroid slices and thyroid cell particulate fractions.

These compounds reach their site of action in the thyroid within minutes following their administration to rats intraperitoneally. There is no concentration gradient for these compounds in the thyroid versus serum, and their uptake by the thyroid does not require the presence of the pituitary gland.

The experiments with rats demonstrate an interesting degradation of these S^{35}-labeled compounds by the thyroid. The labeled sulfur of thiourea is chiefly converted to S^{35} sulfate.

The minimal concentration of these compounds in the thyroid necessary to inhibit the formation of PBI^{131} by the thyroid is approximately 2.6×10^{-5} *M*.

Acknowledgments

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THYROXINE EXCRETION, A POSSIBLE CAUSE OF GOITER

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TWENTY-FIVE years ago McCarrison (1) described an iodide resistant goiter in rats kept under "unsanitary" conditions or fed soy bean meal. A more thorough report was later presented by Sharpless, *et al.* (2) who showed that rats fed soy meal required two times more dietary iodide to prevent goiter than control rats. It was suggested but not demonstrated that these goiters may have been due to a thiocyanate-type substance in the soy meal. Wilgus, *et al.* (3) and Halverson, *et al.* (4) have amply confirmed the goitrogenic action of soy flour. Astwood (5) observed that the goiter in soy-fed rats exhibited a very high 6-hour uptake of I^{131}. This latter observation reduced the likelihood of thiocyanate or propylthiouracil type goiters which, typically prevent retained uptake of I^{131}.

At present, all known goitrogenic substances (except thyrotrophic hormone) interfere with thyroid function and cause decreased uptake of I^{131}. Soy bean flour, therefore, must be different since it produced a high uptake goiter and was not readily inhibited by iodide therapy. These data therefore suggest that the iodide requirement of experimental animals was increased by the addition of soy flour to the diet. Two possibilities suggest themselves: (1) interference with absorption of iodide from the intestines; (2) loss of thyroxine by an abnormal route. The first of these possibilities has never been demonstrated, so far as I am aware. We investigated the second possibility because Albert and Keating (6) have shown a very active entero-hepatic circulation of thyroxine (100% of circulating thyroxine was secreted into the gut of rats each hour). It is suggested that a loss of finished thyroxine might have a more devastating effect on the thyroxine reserves of the rat than the loss of a chemically equivalent amount of iodide. Van Middlesworth (7) has shown that goitrogenic cereal diets result in loss of 70% of the dietary iodide as organic iodide in the feces and this fecal iodine was of thyroid origin. Therefore, we investigated the possibility of soy flour causing an increased fecal thyroxine excretion.

METHODS

Thirty-day old male rats were fed various diets for 2–4 days, then injected intraperitoneally with 10–15 μc. I^{131} thyroxine[2] (1–2 μg. *l*-thyroxine) and the excreta were

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[2] Obtained from Abbott Laboratories, Chicago, Illinois.

TABLE 1. HIGH UPTAKE GOITER IN RATS

Diet	No. Rats	I Content μg./kg.†	Fed diet 2 wks.		Fed diet 3 mos.	
			Thyroid			
			mg.* %	I^{131} % dose 6 hr.	mg. %	I^{131} % dose 6 hr.
Gluten-Sucrose	8	90	9	26		
Sucrose	8	100	9	35		
Starch	15	70	10	30	10	60
Soy	12	170	14	38	15	84
Wheat	6	?	12	56		

* Mg. thyroid per 100 gm. body weight.
† Analysis by chloric acid digestion (Zak *et al.*, *Anal. Chem.* **24**: 1345 (1952) and by equilibrated specific activity method (7).

collected separately from metabolism cages. The daily feces and urine I^{131} were added progressively and expressed as accumulative fecal I^{131} in terms of per cent of injected I^{131} thyroxine. Finally, the plasma, thyroid and total body I^{131} were determined as percentage of the injected thyroxine.

All diets were prepared with 26% casein,[3] a complete salt and vitamin mixture, 10% cotton seed oil, and the remaining 60% was composed of the additives shown in Table 1.

RESULTS

Table 1 shows our results on the high-uptake goiter in rats. The goiter produced by this soy flour diet was small but significant ($P = 0.001$) and the 6-hour uptake of I^{131} was clearly as high or higher than controls. After animals had been fed the soy diet and starch diet for three months, the same degree of goiter was shown in the soy flour rats but the I^{131} uptake was much higher in these animals.

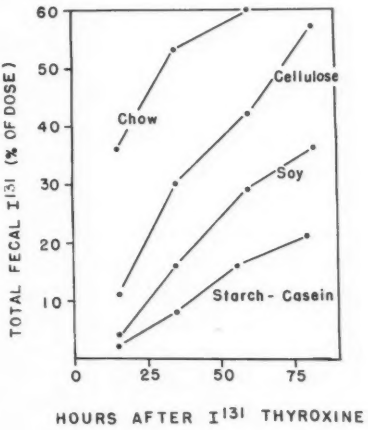

FIG. 1

Figure 1 illustrates the accumulated thyroxine excretion in the feces of rats fed different diets. The resultant fecal loss of thyroxine in soy flour fed rats was almost twice that of control animals after $3\frac{1}{2}$ days and always exceeded the control values. Since the soy fed animals persistently had twice the fecal mass of control rats, it was considered that fecal mass may be partly responsible for this increased excretion of thyroxine. Therefore, 30% purified cellulose was added to the con-

[3] Vitamin-free casein, obtained from Nutritional Biochemical Company, Cleveland, Ohio.

trol diet. The animals continued to eat the same number of calories each day but fecal I^{131} thyroxine excretion was greatly accelerated in cellulose fed rats (Fig. 1). Effects similar to cellulose were demonstrated by adding 30% bran to the control diet. However, the cellulose and bran-fed rats did not grow as well as control animals.

Interesting additional data were obtained by comparing thyroxine excretion in starch-casein fed rats with thyroxine excretion with rats fed standard laboratory chow (Purina dog chow). Figure 1 shows that so-called "normal" diet, standard laboratory chow, resulted in greater loss of I^{131} thyroxine in 12 hours than control starch-casein fed rats excreted in $3\frac{1}{2}$ days. In fact, the rats fed laboratory chow excreted thyroxine at least 15 times faster than animals fed starch-casein. Since laboratory chow contains

TABLE 2. PLASMA AND CARCASS I^{131} 5 DAYS AFTER I^{131} THYROXINE

Diet	No. rats	PBI^{131} c/s/cc*	PBI μg.%	Carcass I^{131} % dose
Chow	8	1.0		2.2
Soy	6	9.7	3.1	7.0
Control (Starch)	8	13.	4.1	12.

* Plasma PBI^{131} determined in scintillation well counter with background of 0.1 c/s. All animals were tested simultaneously and received the same dose of I^{131} thyroxine. The I^{131} thyroxine standard was not counted in this instrument, therefore the plasma data are not expressed as percentage of injected dose.

large amounts of iodide, it is not goitrogenic, even though it appears as though large demands are made for thyroxine production.

Further studies were undertaken regarding the distribution of I^{131} thyroxine remaining in animals fed these diets. Table 2 summarizes these results. Control animals retain 13 times more I^{131} thyroxine in their plasma than laboratory chow-fed rats and 33% more than soy-fed rats. It is interesting to note that the control and soy diets, which contain similar amounts of iodide, produced the same differential in chemical plasma-protein-bound iodine as was shown with radioactive protein-bound-iodine. Table 2 shows the resulting retention of I^{131} from thyroxine within the carcass (less thyroid and intestine) of control rats was five times greater than chow fed animals 5 days after I^{131} injection and again the carcass of soy-fed rats contained only two-thirds as much I^{131} as control animals.

DISCUSSION

The quantitative importance of fecal thyroxine was shown by Kendall (8). Taurog (9) has summarized extensive studies regarding the nature and source of intestinal thyroxine.

The data presented here are highly suggestive of a previously unrecognized mechanism of goitrogenesis. Loss of thyroxine by way of the feces may so increase the daily requirement of thyroxine that the thyroid gland

may be overtaxed to produce the hormone. The mechanisms by which soy flour, cellulose, bran, or standard laboratory chow increase this fecal loss of thyroxine have not been clarified. There are several possibilities such as (1) mechanical, the fecal mass being so great that fecal contents may move too rapidly to afford reabsorption of biliary thyroxine. (2) The rate of production of bile or concentration of thyroxine in the bile may be altered by certain dietary substances. (3) The capacity of the intestine to reabsorb thyroxine secreted into the intestine may likewise be chemically altered by some substances. (4) It can also be considered that thyroxine remaining in the intestine for prolonged periods may result in bacterial destruction and release of thyroxine iodide more efficiently thereby making that iodide available for reuse by the thyroid. Such a mechanism could increase the utilization efficiency of dietary iodide. It might be expected that low iodine diets would potentiate goiter production by thyroxine depletion and an increased iodine intake could possibly alleviate the goiter.

Finally, these mechanisms make it important to consider liver function and gastrointestinal function as well as the absolute amount of iodine available as possible causes of goiter.

SUMMARY

Rats injected with 1–2 μg. of I^{131} thyroxine have been shown to excrete the thyroxine at rates dependent upon their diets. Soy flour and laboratory chow caused thyroxine excretion rates which were 2 to 20 times greater than control diets.

It is suggested that this mechanism may function to increase the iodide requirements of rats and may sometimes account for a "thyroxine depletion goiter."

Acknowledgment

These investigations were begun in the laboratory of Dr. E. B. Astwood, New England Center Hospital, Boston, Massachusetts. The help, encouragement, and cooperation of Dr. Astwood is gratefully acknowledged.

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A COMPARISON OF THE CARCINOGENIC EFFECT OF INTERNAL AND EXTERNAL IRRADIATION ON THE THYROID GLAND OF THE MALE LONG-EVANS RAT[1]

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THERE has been increasing interest since the first case of thyrotoxicosis was treated with I^{131}, in the possible carcinogenic effect of this form, or indeed any form, of irradiation of the thyroid gland. Evidence bearing on this possibility has been sought in various ways. Surveys have been made of patients, mostly adults, whose thyroidal disorders have been treated by radiation (1). Surveys have also been reported of the incidence of thyroid malignancy in patients who, as children, had received irradiation (2). Experimental series, comparable to the one to be presented, with somewhat conflicting results have also been published (3, 4, 5, 6, 7 and 8).

In view of the possible significance of the first of these direct experiments with I^{131} (3) it was decided to repeat the work but also to extend the observations to compare the effect of internal and external irradiation.

MATERIALS AND METHODS

In our series 168 three-month-old male rats of the Long-Evans strain were used. They had been raised and were maintained on a low iodine diet (Stock diet #1: Taurog and Chaikoff, 1946) (9). The diet consists of 68.5% soft wheat, 5% casein, 10% alfalfa, 1.5% sodium chloride, and 5% sardilene, a fish oil concentrate containing 400 AOAC chick units of vitamin-D and 3000 U.S.P. units of vitamin-A per gram.

The animals were divided into three groups using a table of random numbers. The groups contained an approximately equal number of rats and were: a control group, a group of I^{131}-treated rats given a single intraperitoneal injection of 400 μc. (N.Y.C. standard millicurie), and externally irradiated rats. This third group received a calculated tissue dose of 900r given in three doses of 300r each delivered in 5 days. This dose was chosen, not as an estimated equivalent to the I^{131} injection of 400 μc., but rather as corresponding to the effective dose for treatment of thyrotoxicosis in human patients.

The animals were kept in wire cages, generally two or three of the same subgroup in the same cage, with food and water available at all times. They were examined and

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weighed periodically. Particular attention was paid to the animals' coats and general appearance including vigor or weakness, activity or sluggishness as indicative of altered thyroid function. Animals that appeared ill or had an obvious neoplasm of any kind, were sacrificed for immediate post mortem examination. Two years later the survivors were sacrificed also by chloroform.

At autopsy the trachea, with the whole thyroid gland, was removed en bloc, fixed in Bouin's fixative, embedded in paraffin and sectioned serially at 10μ, except for every fifth slide for which the ribbon was prepared at 5μ. All viscera were examined grossly. Routine sections for microscopic study were made of the cervical lymph nodes, salivary glands, liver, pancreas, spleen, kidneys, adrenals, and testes. Sections of the heart and of the gastrointestinal tract were prepared only when indicated. Obvious neoplasms and pathological lymph nodes in any location were taken for section. The skull cap was removed and the brain, with the base of the skull, was fixed in 10% formalin for 48 hours. The pituitary gland was then removed and sectioned serially at 5μ. All sections were stained with hematoxylin and eosin. Mucicarmine, Masson's trichrome and Laidlaw's silver reticulin stains were used as indicated.

Inevitably some animals died unnoticed and were often partially eaten by cage-mates before the corpses were discovered. Animals in which post mortem autolysis was severe enough to preclude the accuracy of diagnosis were discarded. Eighteen animals had to be discarded, 3 in the control group, 4 in the I^{131}-treated group and 11 in the externally irradiated group.

During the course of the experiment and before sacrifice, in regular examination of the animals, it was noted some time after I^{131} administration, usually between 2 or 3 months or even longer, that the rats in this group were heavy, sluggish and their coats were sparse, with coarse dry hair. These findings were interpreted as evidence of myxedema. At autopsy of an animal in this condition, marked edema of the supporting tissues was regularly observed, particularly in the subcutaneous and retroperitoneal tissues. Nothing comparable was observed in the controls nor in the x-ray-treated animals, although a brief epidemic of mange resulted in some confusion about the state of the animals' hair.

It was somewhat puzzling to find that in the three subgroups of the I^{131}-treated rats there was a variation in the degree of myxedema. Whether this could have been due to the possible 10% variation in dose or to individual differences in susceptibility or possibly to an error in dose measurement, is not clear. The subgroups were chosen by random selection of litter mates and treated on December 30, 1952, January 29, 1953 and February 26, 1953.

Correlation of the degree of hypothyroidism with partial, often almost complete, ablation of the thyroid gland in the I^{131}-treated rats was not difficult in the animals dying of intercurrent disease. Those, however, who survived until the time of sacrifice seemed to improve somewhat from what was their lowest hypothyroid state. There was, however, no evidence of compensatory regeneration of thyroid tissues at autopsy.

RESULTS

The numbers of animals in each group are shown in Table 1. This also shows the average survival period and the number of thyroid neoplasms found. The histological types of thyroid tumors are shown in Table 2.

It will be noted that the greatest number of tumors in our series occurred in the x-ray-treated rats. Also, the only proven malignant tumor, i.e., proven by regional lymph node metastases, was found in an x-ray-treated animal (Fig. 1). However, a considerable number of tumors were also found

TABLE 1. INCIDENCE OF THYROID NEOPLASMS IN NORMAL LONG-EVANS RATS AND SIMILAR LITTERS TREATED WITH I^{131} OR X-RAY

	Control	I^{131}	X-Ray
Number of rats	53	52	45
Average survival in months	23.0	28.6	22.0
Number of neoplasms	15*	1	25†
Percentage of neoplasms	24.5	1.9	44.5

* 2 animals with 2 tumors.
† 5 animals with 2 tumors.

in the controls. In the I^{131}-treated rats, on the other hand, only one benign adenoma was found. The paucity of neoplasms in the I^{131} group we thought might be related to the extreme degree of atrophy, accompanied by fat replacement, induced by the high I^{131} dosage. This supposition is apparently confirmed by the work of Lindsay, Potter and Chaikoff (4). The number of tumors in their I^{131} rats treated with lower dosage is very striking. Table 3 is a modification of Table 1, page 184, of these authors, shown for purposes of comparison with our figures. It is obvious, therefore, that the thyroid tissue dose of x-ray, 900 r in 5 days, was biologically lower than that delivered to the thyroid with 400 μc. of I^{131}. This is borne out by the fact that there were very few instances of partial ablation of the gland in our x-ray-treated rats, and no x-ray-treated gland was found to be totally ablated.

TABLE 2. CLASSIFICATION OF THYROID NEOPLASMS

	Rats number	Adenomas number	Solid carcinomas number	Follicular and papillary carcinomas number
Control	53	3 (5.7%)	12 (22.6%)	—
I^{131}	52	1*(1.8%)	—	—
X-ray	45	1 (2.2%)	20 (44.4%)	4†(8.8%)

* Papillary.
† 3 Follicular, 1 Papillary.

TABLE 3. INCIDENCE OF THYROID NEOPLASMS IN NORMAL LONG-EVANS RATS AND IN THOSE GIVEN INJECTIONS OF I^{131}*

I^{131} Injected μc.	No. rats sacrificed	Alveolar Ca. incidence (per cent)	Foll. Ca. and Pap. Ca. (No.)
0 (Control)	9	33	0
0 (Control)	31	33	0
0 (Control)	116	30	0
10	6	33	1
25	20	35	3
100	10		1
200	12	8	0
200	4	25	0
400	25		0
400	121	1	0

* Lindsay, Potter and Chaikoff, *Journal of Cancer Research*, April, 1957.

FIG. 1. *P&S44873:* Gross photograph. X-ray-treated rat. Follicular carcinoma which metastasized to a regional lymph node.

As has been seen in our own Table 2 and in Lindsay's Table 3, the neoplasms can be follicular (Figs. 1 & 2) or papillary (Fig. 3), closely resembling their human counterparts. There is, however, one tumor type which Lindsay has chosen to call "alveolar" type but which we prefer to call "solid" type (Figs. 4, 5, 6). This is somewhat reminiscent of some human thyroid neoplasms but it is far more common in the Long-Evans rat thyroid than in the human thyroid. We found no neoplasm of this type in this series which invaded the tissues outside the gland, and although the larger ones were of considerable size we found none which had metastasized. The tumors, however, although circumscribed are not encapsulated, and in the larger ones there is occasionally evidence of vascular invasion. They are frequently found in both lobes and in multicentric foci. The single, very small lesions look quite innocent (Fig. 6), and the interpretation of these as carcinomas derives from the findings of local extension in the gland and the vascular invasion in the larger tumors which are similar in cell type. Mitotic activity is minimal in all of these neoplasms and this was so even in the only one with regional node metastases. The papillary tumors pose the same problem of interpretation as they do in human thyroids. We have classified some as papillary cystadenomas, which might well be thought by other pathologists to represent involutional nodules of non-toxic nodular goiter. One we considered a papillary carcinoma (Fig. 3). This is definitely a true neoplasm, but everyone knows the divergence of opinion as to what features constitute a benign and what a malignant papillary tumor of the thyroid.

Table 4 shows the incidence of neoplasms of other tissues than the thyroid. The lesions of the pituitary are of possible physiological interest, and in 136 pituitary glands suitable for study 4 so-called clear-cell adenomas were found (Fig. 7). It must be acknowledged that the pituitary glands were not studied in all of the cases by serial section, nor were differential cell counts made. As might have been expected these lesions were found in the I^{131}-treated rats, where severe radiation damage had caused depression

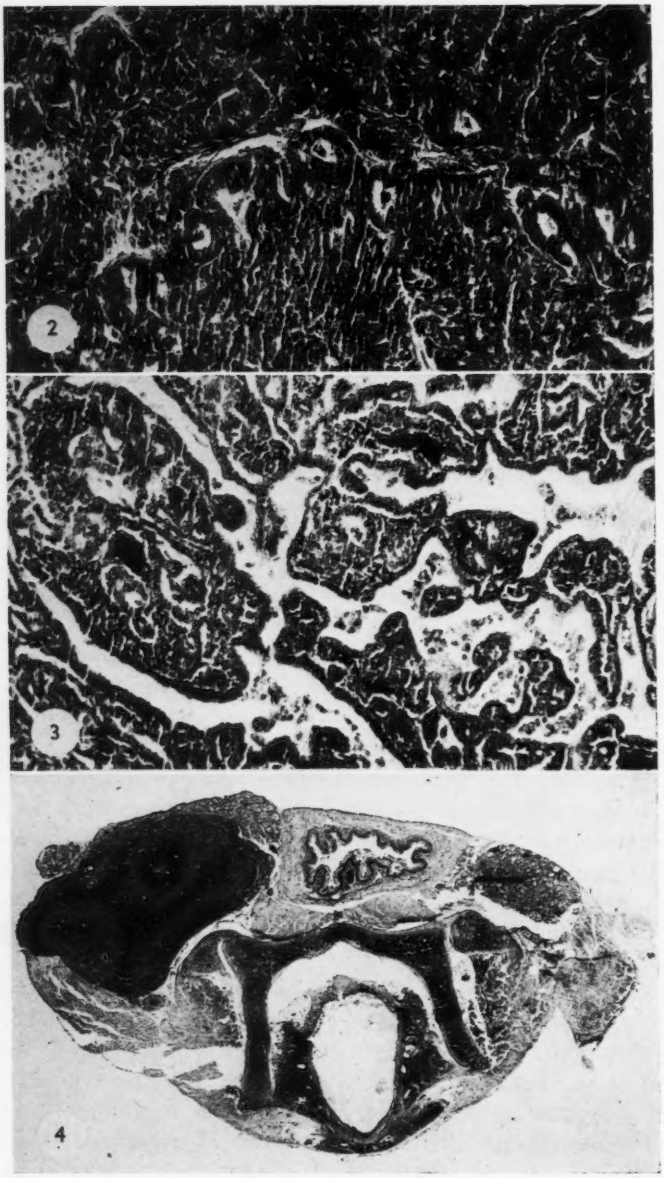

FIG. 2. *P&S 48017:* Photomicrograph. X-ray-treated rat. Infiltrating carcinoma showing follicles.

FIG. 3. *P&S 48134:* Photomicrograph. X-ray-treated rat. Papillary carcinoma resembling the human variety.

FIG. 4. *P&S 49128:* Photomicrograph. Control rat. Solid tumor replacing most of one lobe of the thyroid gland.

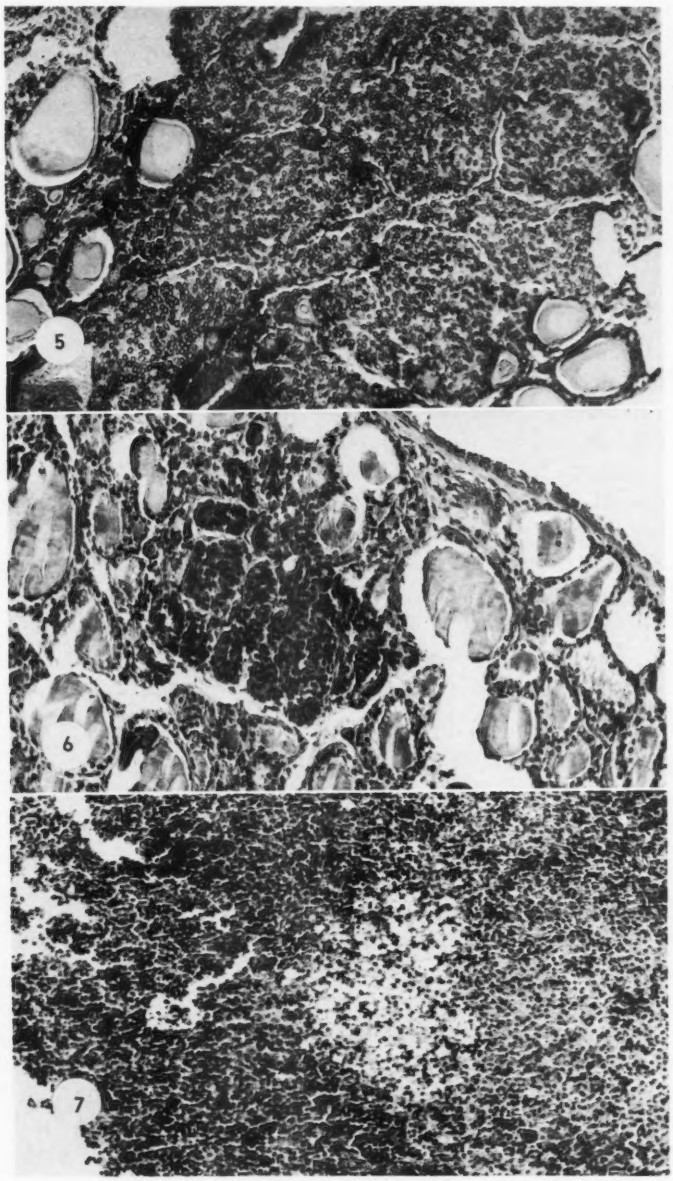

FIG. 5. *P&S 49123:* Photomicrograph. Control rat. Showing the cellular detail of the solid type of spontaneous carcinoma occurring in Long-Evans rats.

FIG. 6. *P&S 47235:* Photomicrograph. Control rat. A single small lesion with a similar cellular structure as the larger solid-type carcinomas, found because of serial sectioning of the whole thyroid gland.

FIG. 7. *P&S 48142:* Photomicrograph. I^{131}-treated rat. Pituitary gland showing a so-called clear-cell adenoma, found on serial section.

TABLE 4. NEOPLASMS IN RAT SERIES IN TISSUES OTHER THAN THE THYROID

	Control	I^{131}	X-ray
Adrenal neoplasms	6	1	6
Parathyroid adenoma	1	2	1
Pituitary adenoma	0	4	1
Pituitary carcinoma (?)	0	0	1
Lymphosarcoma	6	2	5
Fibrosarcoma	3	1	0
Others*	1	1	4
Total	17	11	18

* Rhabdomyosarcoma, Liposarcoma, Adenosarcoma, Interstitial-cell tumor of testis, Hemangiopericytoma, Islet-cell adenoma, Carotid body tumor.

of thyroid function. The single tumor of the pituitary considered malignant occurred in an x-ray-treated rat. The histological interpretation of this tumor is debatable, but it was large and had invaded the sella turcica so that it is at least a locally malignant neoplasm.

The adrenal tumors in these rats are of interest in view of the findings of Moon *et al.* (10) that medullary tumors were found in 10 of 15 rats to whom pituitary growth hormone was administered over a period of 12–16 months, and not in their controls. In our series medullary tumors were found in all groups, including the controls.

SUMMARY

A single intraperitoneal dose of 400 μc of I^{131} in this series of male Long-Evans rats maintained on low-iodine diet induced symptoms of hypothyroidism and partial or almost complete ablation of the thyroid gland. Only one thyroid neoplasm was found in rats so treated. This was a benign follicular adenoma.

X-ray irradiation of similar rats, 900r in 300r doses, delivered in 5 days, did not induce hypothyroidism, nor was atrophy of the gland a striking feature in these animals. A large number of benign adenomas and tumors thought to be carcinomas, designated as the "solid" type, were found in these x-ray treated rats.

The control series, not treated, were found to have neoplasms of these same types, but in smaller numbers than in the x-ray-treated animals.

Papillary and follicular carcinomas were not found in the controls but were found in the x-ray-treated rats. The only malignant tumor with metastases was found in an x-ray-treated rat, a follicular carcinoma with lymph node metastases.

The coexistence of multiple endocrine neoplasms was noted.

Benign and malignant neoplasms in other sites are reported.

Acknowledgment

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EFFECT OF GROWTH HORMONE ON GROWTH IN HYPOPHYSECTOMIZED-PANCREATECTOMIZED RATS

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IT IS well known that growth is retarded in animals deprived of either insulin (pancreatectomy) or growth hormone (hypophysectomy). The relationship between these two hormones in growth is not clearly understood. It has been found that insulin, which can cause some nitrogen retention in the absence of growth hormone (1, 2, 3), is necessary for the anabolic action of the growth hormone (1, 2, 4). Since extra amounts of insulin are needed for the maximal response to growth hormone in cats and dogs (1, 2, 4), it has been suggested that growth hormone exerts some, if not all, of its growth promoting effect by stimulating the secretion of insulin (5).

This paper presents a study of the effect of growth hormone in rats deprived of the pituitary gland and at least 99.5% of the pancreas. Small amounts of insulin were necessary for the control of diabetes in these rats. In contrast to the findings in cats and dogs, additional insulin was not needed for the growth reponse to growth hormone.

EXPERIMENTAL

Female rats weighing 120–145 gm. of the Instituto de Biologia y Medicina Experimental were pancreatectomized and 13–14 days later, hypophysectomized. The method for removing at least 99.5% of the pancreas and the post-pancreatectomy care, as well as the acute effects of hypophysectomy in the "totally" pancreatectomized rat, have been described earlier (6, 7). Complete removal of the whole pituitary gland was achieved in all rats as evidenced at autopsy by the absence of pituitary tissue in or near the sella turcica when examined with a binocular microscope at 10× magnification.

The diet used, #4370, consisted of casein, 20%; L-cystine, 0.3%; dextrin, 66.7%; corn oil, 8%; salts, 4%; and all vitamins needed by the rat (6). An aqueous suspension of the diet with pancreatin added, 10 mg./gm. of diet, was tube-fed twice daily with an interval of six or more hours between meals. The operated rats were never fed *ad libitum*. From the fourth day after pancreatectomy each rat was tube-fed 7 gm. of diet/day, except for two days at the time of hypophysectomy; no food was given on the day of the second operation and only 5.5 gm. the following day. After hypophysectomy insulin

[1] Schering Scholar of the Endocrine Society and Fellow of the John Simon Guggenheim Memorial Foundation for 1955.

TABLE 1. EFFECT OF GROWTH HORMONE (GH) ON BODY WEIGHT GAIN, NITROGEN RETENTION, AND GLYCOSURIA IN HYPOPHYSECTOMIZED-PANCREATECTOMIZED RATS[a]

Regular insulin		Body weight gain, gm./day[b]		Nitrogen retained,[c] mg./day[b]		Urinary glucose, gm./day[b]	
Units/day	No. of days	GH (7)	Control (6)	GH (7)	Control (6)	GH (7)	Control (6)
3	6	2.3±0.3	0.5±0.3	62± 9	14± 6	0.21±0.02	0.10±0.03
1	3	2.6±0.5	0.7±0.4	73± 5	17± 4	0.41±0.05	0.34±0.11
0.3	2	−0.7±5.0	−0.5±1.0	68±16[d]	26±14[d]	0.52±0.07	0.53±0.07

[a] These rats were hypophysectomized 13–14 days after the removal of more than 99.5% of their pancreas. Growth hormone injections, 0.05 mg. twice daily, were begun on the fifth day after hypophysectomy. There were 7 rats in the growth hormone (GH) group and 6 in the control group. All rats were tube-fed 3.5 gm. of food twice daily. Half of the daily dose of insulin was injected immediately after each feeding. The average nitrogen content of 7 gm. of the diet with pancreatin added (10 mg./gm.) was 196 mg.

[b] Mean ±standard error.

[c] The amount of nitrogen retained was determined as the difference between the amount fed and that excreted in the urine and feces.

[d] See text for discussion.

was always injected immediately after each meal. The growth hormone,[2] which was highly purified (8), was dissolved in slightly alkaline 0.85% NaCl solution and was injected intraperitoneally twice daily.

The rats were maintained in individual, heated (26–28° C) metabolic cages. The procedures for collecting urine, feces, and food samples have been described (9). The nitrogen contents of the above were determined by the microkjeldahl method, glucose in urine by Benedict's quantitative method (10), glucose in blood by the method of Haslewood and Strookman (11), and ketone bodies in urine by the qualitative nitroprusside test (Rothera).

RESULTS

Hypophysectomized-pancreatectomized rats when tube-fed 7 gm. of food daily needed 3 units of regular insulin[3] to maintain the urinary excretion of glucose at less than 0.2 gm./day (Table 1 and Fig. 1). Their small body weight gain and nitrogen retention were not statistically significant. Growth hormone administration, 0.1 mg./day, markedly increased the body weight gain and nitrogen retention but had no significant effect on the amount of glycosuria.

Decreasing the dosage of insulin from 3 to 1 unit/day significantly increased the glycosuria of both groups ($P<.05$) but did not reduce the rate of body weight gain or nitrogen retention of the growth hormone-treated rats.

The growth hormone-treated rats receiving one or more units of insulin/day gained at least 2.3 gm./day[4] and retained 34% of the nitrogen fed. If

[2] The growth hormone was obtained from Dr. C. H. Li of the University of California, Berkeley, Calif.

[3] The regular insulin used in this study was kindly supplied by Laboratorios Armour of Buenos Aires, Argentina.

[4] This growth rate is twice that observed in hypophysectomized (male) rats treated

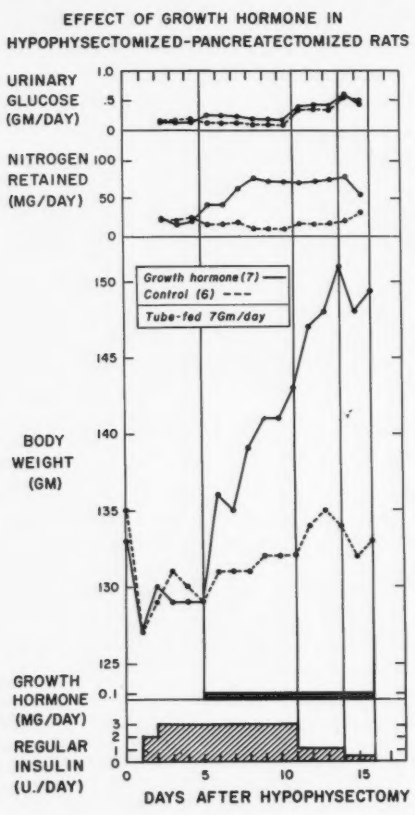

FIG. 1. Effect of growth hormone in hypophysectomized-pancreatectomized rats. Their average daily nitrogen intake was 196 mg.

all of the nitrogen was retained as muscle protein, the increase in muscle tissue would be equal to 83% of the body weight gain. The ratio of water to nitrogen is 24.4 in the thigh muscle of hypophysectomized rats (12).)

When the insulin was reduced to 0.3 unit/day, both groups of rats excreted slightly more glucose in the urine, lost body weight, and many rats became listless and cold. There was no decrease in the volume of urine excreted during this period except in one animal prior to death. One rat of each group died during this two-day period and two more of each group died during the subsequent three days even though the insulin dosage was increased to 3 units/day. None had ketonuria. The blood glucose level prior to death in the few animals tested ranged from 800 to 1,200 mg./100 ml. (In an earlier study the hypophysectomized-pancreatectomized rats died within 48 hours with blood glucose levels of 1,370 mg./100 ml. when insulin was withheld and tube-feeding was continued (7).) In view of the high mortality rate during and after this period, the failure of the rats to gain weight and the distended stomachs[5] found in many of the rats, it is very doubtful that the retention of nitrogen observed during this period is significant (Table 1).

DISCUSSION

Insulin is necessary for the control of diabetes and survival of hypophysectomized-pancreatectomized rats when they are tube-fed (7). In these

with the same amount of growth hormone and tube-fed the same amount of food, 7 gm./day. The hypophysectomized rats did not receive insulin or pancreatin and they were maintained, all in one cage, in another part of the institute.

[5] Gastric retention is one of the signs of severe diabetes in the rat (6), even in those hypophysectomized.

animals additional insulin is not needed for the growth promoting action of growth hormone (Table 1 and Fig. 1), which is in contrast to the findings in the cat and dog (1, 2, 4). The most likely explanation for this difference is that the rat is not as sensitive to the diabetogenic action of growth hormone (13) as are the cat and dog (5). In the latter species the additional insulin is probably necessary for the control of the exacerbation of the diabetes following the growth hormone administration.

It was observed recently that hypophysectomized rats tube-fed extra food gained body weight and that 67% of the increment was fat (9). These findings are similar to those of Salter and Best (3) who found that hypophysectomized rats treated with insulin gained weight only if they ate extra food. On the other hand, extra food was not necessary for the growth promoting action of growth hormone in hypophysectomized (9) or hypophysectomized-pancreatectomized rats (Table 1 and Fig. 1); the dosage of insulin given to the latter was the same as that used by Salter and Best. Another difference between these two groups of experiments is that 15–17% of the weight gain was protein in the rats given growth hormone whereas in the others it was only 9%.

These findings support the suggestion of Krahl (14) that in the formation of peptides the role of insulin is to make energy available and the role of growth hormone is to direct this energy toward peptide synthesis. In the absence of the pituitary hormone, this energy would be stored mostly as fat (9, 15, 16). If in the latter situation sufficient energy is available, a small amount of nitrogen may be retained, as seen in force-fed (9, 16) and insulin-treated (3) hypophysectomized rats. It is not known, however, how much of the protein formed under these conditions can be accounted for by the increase in the size of the "metabolic" organs and the fat depots (3, 9, 16). Keratin synthesis may also account for a large part of the retained nitrogen since hair growth continues in the absence of the pituitary gland if sufficient food is given (9). On the other hand, increased food intake has essentially no effect on the impaired growth of skeletal muscle and bone in animals deprived of growth hormone (9, 16).

SUMMARY

Hypophysectomized-pancreatectomized rats needed 3 units of regular insulin daily for the control of diabetes when tube-fed 7 gm. of food per day. They maintained their body weight constant and retained no nitrogen. When treated with growth hormone (0.1 mg./day) they gained 2.3 gm. daily and retained 34% of the nitrogen fed; 17% of the body weight gain was protein. Extra food and insulin were not necessary for the anabolic effect of growth hormone. There was no effect of growth hormone on glycosuria. Half of the rats in each group died when the insulin dosage was reduced to 0.3 unit/day.

Acknowledgments

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NOTES AND COMMENTS

RECOVERY OF HUMAN PITUITARY GONADOTROPIN FROM DILUTE URINE

The extraction of human pituitary gonadotropin (HPG)[1] from urine is best accomplished by adsorption on kaolin and elution with appropriate alkali. This method is now widely used in clinical endocrinology laboratories and gives promise of becoming a standardized procedure [cf. reviews by Albert (1), Loraine (2)]. Although many aspects of the preparation of urinary HPG concentrates for biologic assay have been studied, several technical details have remained unexplored. One of these details concerns the recovery of HPG from dilute urine, obviously an important problem when clinical assay of HPG is needed for patients with diabetes insipidus or for patients who have a large volume of urine for reasons other than diabetes insipidus. The only reference we have found to the recovery of HPG from dilute urine is in an addendum to a paper by Crooke and associates (3), who wrote: ". . . Dr. P. S. Brown, in this laboratory, has observed that in very dilute urine adsorption of gonadotropin onto kaolin may be impaired." The addendum also stated that this subject would be discussed in a subsequent report. Since 3 years have elapsed and no documentation of this statement has appeared to our knowledge, it was thought worth while to investigate the efficacy of the kaolin method in recovering HPG from dilute urine. The investigation was made from three approaches:

1. EVIDENCE FROM ROUTINE CLINICAL ASSAYS

Search through our records of routine clinical assays for HPG disclosed reports on 12 patients whose urinary volume per 24 hours was between 5 and 10 liters. HPG assays were negative in these instances. However, clinical evidence of hypopituitarism and laboratory evidence of hypopituitarism on grounds other than the results of the gonadotropin assay were present in all cases. Thus, in each instance the negative HPG assay could be explained by lack of pituitary gonadotropic function. We have no information on HPG assays (kaolin method) in patients who had large urinary volumes from diabetes insipidus or other causes and who had clinical and laboratory evidence of normal gonadotropic function. Critical evidence, therefore, from routine clinical HPG assays is not available to illuminate the problem of recovery of HPG from dilute urine.

2. EVIDENCE FROM SURGICAL HYPOPHYSECTOMY

A previous study from this laboratory (4) disclosed that HPG disappeared from the urine in a mean time of 5.8 days after surgical removal of the pituitary gland of patients with mammary cancer. The data of the table in this study were re-evaluated for the purpose of the present paper as follows: For each of the eight patients, the mean of the preoperative HPG assays was determined and assigned a value of 100 per cent. After hypophysectomy, the daily titer of each patient was expressed as a percentage of the preoperative mean. The means of all such calculations are shown in the figure. The curve of disappearance of HPG resembles an exponential curve with a half time of 1.5 days. Also plotted in the figure is the mean urinary volume of the eight patients for each day

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[1] Since the evidence now seems conclusive that the gonadotropic material(s) in urine of men and nonpregnant women who do not harbor tumors producing chorionic gonadotropin is of pituitary origin, it is proposed that the term "human pituitary gonadotropin (HPG)" be used to distinguish this material from human chorionic gonadotropin (HCG).

after hypophysectomy. The preoperative value is the mean of the means of the five preoperative daily volumes for each patient. For the first two days after hypophysectomy, the disappearance of urinary gonadotropin was inversely proportional to the increase in urinary volume. After this time, however, HPG continued to fall while the urinary volume was more or less stabilized between 2 and 3 liters per day. Thus, evidence from surgical hypophysectomy in man would indicate that the behavior of HPG is independent of concomitant changes in urinary volume. However, the possibility remains that during the first few days after operation the disappearance curve of HPG may result not only from removal of the source of HPG but also from the additional complication of impaired recovery of HPG.

3. EVIDENCE FROM EXPERIMENTS ON ARTIFICIALLY DILUTED URINE

A total of 192 liters of male urine, collected as previously described (1, 5), was used in the following experiments. Control aliquots of urine were processed by the procedure described previously (6), using automatic equipment.

Experiment 1.—Two 3-liter aliquots of urine were removed from the male pool. One aliquot was processed as control and assayed on two rats at a dose of 1.5 liters of urine per rat, a volume of urine corresponding to an average 24-hour specimen. The second aliquot was diluted with 7 liters of tap water, yielding 10 liters of diluted urine. The pH of this dilute urine was adjusted to 4.5, 20 gm. of kaolin (the amount used in routine assays and in the control aliquot) were added and, after mixing, the kaolin was allowed to settle overnight. The next morning, the supernatant fluid was siphoned off to a level of 3 liters. The kaolin bed was then agitated, and the 3-liter suspension was processed by the same method as the control aliquot. The respective HPG concentrates from the control and experimental aliquots were assayed simultaneously. This experi-

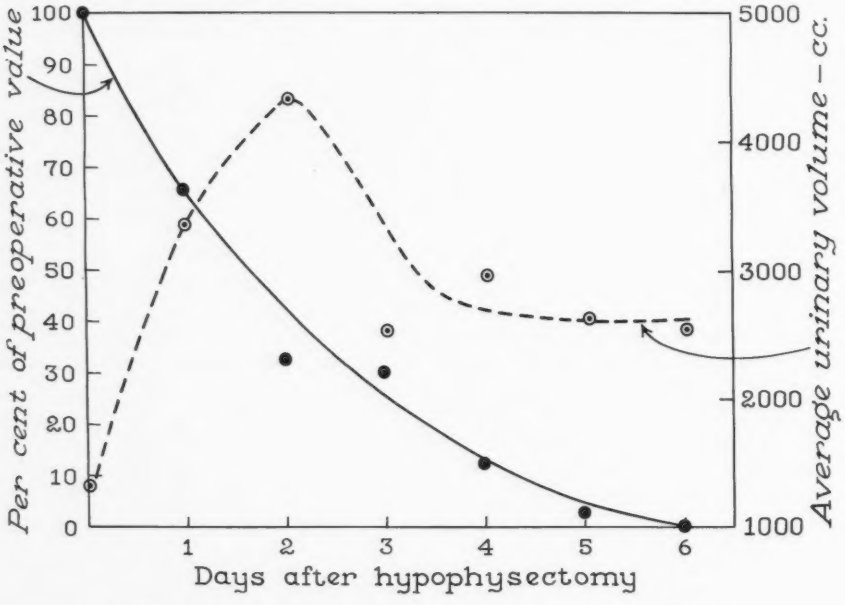

FIG. 1. Behavior of urinary gonadotropin after hypophysectomy. (Average of eight patients.)

ment, therefore, tests the ability of kaolin to recover HPG from 1.5 liters of urine in comparison with its ability to recover HPG from the same amount of urine diluted to 5 liters.

Eight such experiments were carried out. The mean of the eight control aliquots was 14.8 units, and that of the experimental aliquots was 13.4 units. The difference of 1.4 ± 4.2 (standard error) units is not significant. This same experiment was repeated eight more times on another batch of male urine. The mean control value was 15.2 units, and the mean value for the experimental aliquots was 16.7 units. The difference of 1.5 ± 2.1 units is not significant.

Although an occasional patient with diabetes insipidus may have a urinary volume as high as 20 to 30 liters per day, the usual urinary volume, at least in the 12 patients reported in section 1, is between 5 and 10 liters per day. Accordingly, two 3-liter aliquots were removed from the male pool. One aliquot was processed in routine fashion as a control, and the other 3-liter aliquot was diluted to 17 liters with tap water. The same procedure just described was followed. In effect, this experiment tests the ability of kaolin to recover HPG from 1.5 liters of urine when this volume of urine is so artificially diluted as to be contained in a volume of 8.5 liters. The means of four such experiments were 20.3 units for the control and 10.5 for the experimental aliquots. The difference of 9.8 ± 4.9 is statistically significant, since the mean is twice its standard error.

Experiment 2.—Since recovery of HPG was reduced by half from urine artificially diluted to a volume of 8.5 liters, two obvious factors—concentration of kaolin and the specific gravity—both altered by dilution, were studied. Twenty-four liters of male urine were thoroughly mixed and divided into eight 3-liter aliquots. Four of these eight 3-liter aliquots were processed and assayed in the usual fashion. Each of the remaining four 3-liter aliquots was diluted to 17 liters with tap water, and 60 gm. of kaolin (three times the amount used for the control aliquots) were added to each 17-liter volume. After stirring and settling, 14 liters of supernatant fluid from each of the four vessels were siphoned off and the remaining 3 liters were processed in the usual way after suspension of the kaolin. The mean for the control aliquots was 20.3 units, and that for the experimental was 14.7 units. The difference of 5.6 ± 3.6 units could have occurred by chance.

Experiment 3.—Since increasing the amount of kaolin is technically not desirable in processing HPG in dilute urine, experiment 3 was designed to test the role of specific gravity on recovery of gonadotropin. In the first part of the experiment, 24 liters of the male pool were thoroughly mixed and separated into eight 3-liter aliquots. Four of these aliquots were processed in ructine fashion. Each of the remaining four aliquots was diluted to 17 liters with tap water, and 350 gm. of sodium chloride were added to each aliquot to raise the specific gravity to 1.026—the value of the urine before dilution. Sixty grams of kaolin (three times the usual amount) were added, and the procedure was carried out as described in experiment 2. The mean control value was 20.8 units, and the mean experimental value was 16.2 units. The difference of 4.5 ± 6.2 is more favorable statistically to the assumption that the increased kaolin concentration is more effective at an artificially created higher specific gravity.

In the second part of the experiment, the recovery of HPG from diluted urine was determined after raising the specific gravity of the dilute urine, but without increasing the kaolin concentration. Twenty-four liters of male urine were separated into eight 3-liter aliquots. Four of these aliquots were processed as controls, and the remaining four aliquots were diluted to 17 liters each with tap water. Four hundred and fifty grams of sodium chloride were added to each of these aliquots of diluted urine to raise the specific gravity to that of the undiluted specimen (1.025). Twenty grams of kaolin (the amount of kaolin used in the control aliquots and in routine assays) were added, and the procedure described above was carried out. The mean value for the control aliquots was

20.2 units and that for the experimental 17.8 units. The difference of 2.5 ± 3.8 units shows that no significant difference in recovery could be detected.

SUMMARY

The recovery of HPG from urine artificially diluted with water is impaired when the urine is diluted to represent a volume of more than 5 liters per day. Essentially full recovery of HPG from such diluted urine can be effected by the routine method if sodium chloride is added to raise the specific gravity to about 1.025.

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GROWTH HORMONE AND VITAMIN D IN RELATION TO GROWTH AND CALCIFICATION ON A CALCIUM DEFICIENT DIET[1]

The findings of Herting and Steenbock (1) on the beneficial role of vitamin D in promoting growth and calcification in rats kept on a low-Ca diet raised the question if there might be some parallelism in the reactions induced by vitamin D and those induced by the growth hormone. In our presently reported work we have sought to determine if growth hormone could substitute for the vitamin in the production of some of the effects on growth and Ca and P metabolism. Studies were also undertaken to determine if growth could be induced in the hypophysectomized rat by the addition of vitamin D to a low-Ca diet.

METHODS

The basal diet used was the vitamin D-free semisynthetic diet of Bellin and Steenbock (2). Ca was present in trace amounts (0.015%), with inorganic phosphate raised to a level of 0.3% P by additions of an equimolar mixture of KH_2PO_4 and K_2HPO_4. Vitamin D was given orally as 75 I.U. of crystalline vitamin D_2 in 0.1 ml. of cottonseed oil once every 3 days. Some rats were given a daily intraperitoneal injection of 1 mg. of crystalline growth hormone in 0.5 ml. of physiological saline. Others were given an equivalent amount of saline. The rats were young male albino rats (Sprague-Dawley), weighing about 80 gms. Eight rats were assigned to each

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experimental groups. They were housed individually in wire metabolism cages and transferred to clean feeding cages 3 times daily for one hour. Food was available to them *ad libitum* during these periods. Ca and P balances were obtained on individual rats and averaged for collections from the 4th through the 15 th day of the experiment. On the 15th day, blood was collected from the carotid artery for Ca and P analyses by the methods of Wang (3) and Fiske and SubbaRow (4) respectively. The urine and feces were wet-ashed by nitric-perchloric acid digestion.

In the experiments with hypophysectomized animals, the rats, after operation, together with an equal number of controls were given the basal diet supplemented with adequate amounts of Ca (0.5%) and P (0.3%) for 4 days. Following this, all were placed on the low-Ca diet for 21 days for a determination of growth, bone ash and serum Ca.

RESULTS

The results (Table 1) demonstrate that both growth hormone and vitamin D increased not only body weight and weight of organic bone, but bone length as exemplified by the femurs. In their absence this did not occur. When only the growth hormone was given, the gain in body weight averaged about 40% of that induced by vitamin D. When both were given, a distinct additive effect on growth was not demonstrable.

TABLE 1. THE EFFECT OF ADDITIONS OF GROWTH HORMONE AND VITAMIN D TO A LOW-Ca DIET ON GROWTH, BONE ASH, METAPHYSEAL WIDTHS, AND SERUM Ca AND P*

Vitamin D	Growth hormone	No. rats	Increase in body weight	Femur				Metaphyseal widths	Serum	
				Length	Organic	Ash			Ca	Inorganic P
			gm.	mm.	mg.	mg.	%	mm.	mg. %	mg. %
0	0	7	0±4**	24.6±0.4	67.8±2.7	60.2±3.6	47.0	0.1	5.9 (3)	9.0 (2)
0	+	7	+19±8	25.9±0.5	79.1±3.1	66.8±1.4	45.8	0.4	6.0 (4)	9.3 (2)
+	0	5	+56±6	26.9±0.2	77.8±5.6	65.9±3.2	45.9	0.3	10.4	11.3 (3)
+	+	5	+58±5	27.2±0.4	82.9±5.6	64.1±2.1	43.8	0.3	10.4	10.3 (4)

* Averages after 15 days on the diet from 5 to 7 rats in a group except when designated by lesser numbers in parentheses.
** Standard deviation.

Concordant with the evidence for growth, given by the increase in length of bone, the widths of the metaphyses of the wrist bones were greatly increased and to about the same extent by both the growth hormone and by vitamin D. Again there was an absence of an additive effect.

The P balances in general reflected the relative increases in growth. All groups absorbed P readily. When growth hormone was given, the amount of P retained increased about three-fold (Table 2). When vitamin D was given, it was increased nearly five-fold. When both were given, only a small further increase occurred.

The Ca balances, by way of contrast with the P balances (Table 2), show that only vitamin D was effective in reducing the loss of body Ca on the low-Ca diet. The absorption of Ca, in general, followed the respective levels of retention. Calcium was absorbed only by the groups which were given vitamin D. In fact, in the absence of vitamin D, not only was Ca not absorbed, but actually more appeared in the feces than was in-

TABLE 2. EFFECT OF GROWTH HORMONE AND VITAMIN D ON Ca AND P BALANCES

Vitamin D	Growth hormone	No. rats	Mean values (mg./rat/12 days)*									
			Intake		Urine		Feces		Absorbed		Balances	
			Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
0	0	7	8	187	14	143	12	17	−4	+170	−18±2**	+27±12
0	+	7	11	257	15	162	15	19	−4	+283	−19±5	+76±19
+	0	5	18	375	15	226	7	23	+11	+352	− 4±3	+126±13
+	+	5	18	370	15	215	6	23	+12	+347	− 3±2	+134±16

* 4th–15th day on the diet.
** Standard deviation.

gested. The administration of growth hormone had no effect on Ca absorption or retention.

The respective effects of the vitamin and hormone on calcification of bone are presented in Table 1. Both the vitamin and the hormone increased the ash content of the femurs, but while the increase with vitamin D corresponded to the increase in Ca retention, the increase with growth hormone is not in accord with the effect on Ca balances. Apparently, the internal shifts in body calcium well-known to occur when vitamin D is given with mineral deficient diets (1, 5) are different from those which occur with growth hormone.

The vitamin and the hormone were also found to be dissimilar in their respective

TABLE 3. COMPARATIVE EFFECT OF VITAMIN D IN INTACT AND HYPOPHYSECTOMIZED RATS ON A LOW-Ca DIET*

Vitamin D	Pituitary	No. rats	Increase in body weight	Femur				Serum Ca
				Length	Organic	Ash		
			gm.	mm.	mg.	mg.	%	mg. %
0	intact	4	+6±6**	26.8±0.5	8.30±2.8	78.8±3.9	48.6	6.9
0	removed	4	−7±4	24.4±0.8	71.5±9.9	61.8±3.3	46.6	7.4 (2)
+	intact	4	+105±7	30.6±0.3	100.1±7.1	82.1±2.8	45.0	11.7
+	removed	4	+5±4	23.7±0.5	63.8±3.8	58.7±1.9	47.9	11.8 (2)

* Averages after 21 days on the diet from 4 rats in a group except when designated by lesser numbers in parentheses.
** Standard deviation.

effects on the levels of Ca and P in the blood. Whereas vitamin D increased both (Table 1), growth hormone was found to be without effect.

After hypophysectomy, vitamin D was unable to induce growth, to increase the amount of bone ash or to increase metaphyseal widths (Table 3). However, it did increase serum Ca. In this latter regard it was as effective in the hypophysectomized as in the normal rats.

SUMMARY

With rats on a low-Ca (0.015%) normal-P (0.3%) semisynthetic diet both growth hormone and vitamin D increased body weights, femur lengths, femur organic matter, and metaphyseal widths. While P balances generally increased with this increase in growth, the loss of Ca which was decreased by vitamin D, was unaffected by growth hormone. Only vitamin D increased the absorption of Ca; and only vitamin D increased serum Ca and serum inorganic P. A beneficial effect of growth hormone on Ca metabolism *per se* was not demonstrated by our technique. With hypophysectomized rats, as compared with normal rats, the only similarity in the effect of the vitamin was that it increased serum Ca in both. In these the vitamin was unable to induce growth or calcification.

Acknowledgments

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PROGESTIN IN MOUSE EMBRYOS, PLACENTAE, AND AMNIOTIC FLUID[1]

Levels of progestin in the plasma of mice of the A strain on each day of pregnancy have been reported, as has indirect evidence for the production of placental progestin during the latter part of pregnancy (2). Progestin levels in embryos, placentae, and amniotic fluid of mice of the CHI strain can now be described.

Adult female CHI mice in breeding boxes were inspected each morning for vaginal plugs. The day on which a plug was found was designated as day 0 of pregnancy. (On this basis, the CHI mouse delivers on day 19.) On the appropriate days, 19 pregnant mice were killed, embryos and placentae were removed promptly, umbilical cords and amniotic sac remnants were detached, and embryos and placentae were blotted lightly. Five to ten placentae from each mother were weighed on a torsion balance, then ground with washed sand in a mortar and pestle. The resulting paste was shaken up with several volumes of acetone and stored at 4° C. Embryos from each mother to give a total weight of 600–1200 mg. were also weighed, ground with sand, shaken up with acetone, and refrigerated. In addition, fluid was aspirated from the amniotic sacs of six mice before the sacs were opened, totals of 0.12–0.94 cc. being collected. Two newly born (day 19) mice were also weighed, ground, and extracted.

Ground embryos, ground placentae, and amniotic fluid samples were extracted with acetone and ether according to a standard procedure previously applied to plasma samples, and bio-assay of the "free" fractions was conducted in the usual way (3, 4). It has been demonstrated that the bio-assay detects not only progesterone but its metabolites, Δ^4-3-ketopregnene-20α-ol and Δ^4-3-ketopregnene-20β-ol (5); the term "progestin" is applied collectively to these compounds. Bio-assay results are expressed in *Progesterone Equivalents;* one equivalent is defined as equal to the *activity* of 1 μg. progesterone when the latter is assayed by the procedure of Hooker and Forbes in the absence of estrogen and other interfering factors (1).

Assay results for free progestin are presented in Table 1. Values per cubic centimeter of amniotic fluid and per gram of embryo and placenta are actual, while values for total embryos and total placentae are calculated from average weights of embryos and placentae from the corresponding mother. The placentae weighed roughly 110 mg. each, regardless of the day of pregnancy, while the embryo weight ranged from 107–135 mg. on the 13th day to 1340 and 1430 mg. for the two newly born mice on day 19.

"Free" progestin was found in 14 of 21 embryos and newborn mice on days 13–19, inclusive, in 14 of 19 placentae on days 13–18, inclusive, and in 5 of 6 amniotic fluid samples. No consistent correlation was observed between the day of pregnancy and the amout of progestin, or even its presence or absence, in embryos, placentae, and amniotic fluids. Present data do, however, reveal for the first time that progestin is

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TABLE 1. "FREE" PROGESTIN LEVELS IN LATE PREGNANCY IN CHI MICE

Day of pregnancy	Progesterone equivalents per				
	Gm. of embryo	Total embryo	Gm. of placenta	Total placenta	Cc. amniotic fluid
13	0.9	0.1	1.1	0.1	0.5
13	<0.1	<0.01	0.2	<0.01	
13	3.4	0.5	1.4	0.15	1.1
14	0.3	0.05	<0.2	<0.02	
14	0.4	0.1	0.3	0.03	
14	<0.3	<0.06	<0.2	<0.02	
15	1.3	0.5	0.2	0.02	
15	<0.1	<0.03	0.2	0.02	
15	1.5	0.6	1.1	0.12	0.2
16	<0.1	<0.07	1.7	0.19	0.5
16	0.1	0.04	<0.2	<0.02	
16	<0.1	<0.07	0.6	0.11	
17	1.7	1.53	1.0	0.12	<1.6
17	<0.1	<0.07	1.6	0.18	
17	0.8	0.62	1.0	0.11	
17	0.3	0.21	0.3	0.04	1.3
18	0.1	0.09	<0.2	<0.02	
18	<0.1	<0.12	0.6	0.07	
18	0.4	0.30	0.5	0.06	
19	0.5	0.67			
19	0.4	0.57			

usually present in embryos, placentae, and amniotic fluid on days 13 through 18 of pregnancy in the CHI mouse.

SUMMARY

Progestin in variable amounts was usually present in embryos, placentae, and amniotic fluid of mice of the CHI strain on days 13 through 18 of pregnancy. It was also present in two newborn mice.

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THE EFFECT OF PITUITARY TRANSPLANTS ON THE ESTROUS CYCLES OF HYPOPHYSECTOMIZED HAMSTERS[1,2]

Pituitary transplants, except those reported by May (1), have not supported estrous cycles when placed in sites other than the sella turcica (see Harris (2) and Benoit and Assenmacher (3) for reviews). Such transplants have been made in hypophysectomized rats and guinea pigs. It was thought to be of interest to transplant the pituitary into the hamster cheek pouch thus testing a different animal and site for pituitary gonadotrophic activity.

MATERIALS AND METHODS

From one to 5 pituitaries from newborn hamsters of both sexes were transplanted on the day of birth to the cheek pouches of each of 48 female hamsters. The hosts ranged in age from 30 to 163 days. Thirty-three of the 48 animals were hypophysectomized by the parapharyngeal approach prior to grafting, while the remainder were hypophysectomized at varying periods of time after grafting. Daily vaginal smears (6 days per week) were made on all of the animals and the criteria of Ward (4) were used to determine the phase of the estrous cycle. In those animals in which estrus or estrous cycles reappeared after hypophysectomy and grafting, the grafts were removed at periods from 123 to 290 days after transplantation. After graft removal, vaginal smears were continued for 21 days. At the conclusion of the experiment grafts were recovered when present, from the remainder. All grafts were fixed in Zenker-formol, sectioned serially at 5μ and stained by the periodic acid-Schiff method (PAS) according to Wilson and Ezrin (5). Alternate sections of one graft were stained with aldehyde-fuchsin (AF) (Halmi (6)). Ovaries were removed, sectioned at 10μ and stained with hematoxylin-eosin. To check the completeness of the hypophysectomies, the pituitary regions were sectioned at 10μ after decalcification and stained with PAS. The volumes of the anterior lobe in the grafts and the fragments remaining in the hypophyseal fossae were calculated by planimetric methods. Comparisons were made with the volume of the normal adult female anterior lobe, which was calculated as averaging 1.4 mm.^{3}, and the newborn's anterior lobe whose average volume was 0.131 mm.^{3}.

RESULTS AND DISCUSSION

The results of the study are summarized in Table 1 where the animals have been arranged in groups on the basis of the presence or absence of cycles and the amounts of pars distalis found in the grafts and remaining in the hypophyseal fossae. The figures represent the per cent volume of the normal adult female pars distalis.

Neither regular estrous cycles nor estrus appeared in any of the animals without pars distalis fragments, thus confirming in hamsters the results reported for other species (cf. Harris and Jacobsohn (7)). However, the fact that regular estrous cycles appeared in Group I, but were absent after graft removal, suggests the possibility that these grafts had some gonadotrophic activity. Groups II and III also suggest this: the two animals of Group II had estrus occasionally before graft removal and no estrus thereafter, while in Group III regular cycles were replaced by occasional estrus. In contrast with the 7 animals of Groups I, II, and III are the 11 animals of Group VI which had grafts and pituitary remnants, yet remained in diestrus. The ovaries of animals with pituitary fragments at the end of the experiment had follicles without antra and atretic follicles but no corpora lutea. The animals of Groups III and IV had normal appearing ovaries while in the completely hypophysectomized animals the ovaries were atrophic.

Histological examination of the grafts of the 25 animals with successful transplants revealed that 48% were composed of large "chromophobe" cells without PAS-positive

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TABLE 1

Group	Cycles		Range of % vol. of p.d.* in grafts	Range of % vol. of p.d.* fragments in fossa
	Before graft removal	After graft removal		
I (3 animals)	Regular	0	6.7–12.6	0.9–10.7
II (2 animals)	Irregular	0	4.0– 9.4	0.1– 0.6
III (2 animals)	Regular	Irregular	6.3–30.1	12.3–14.2
IV (1 animal)	Irregular	Irregular	0	37.1
V (7 animals)	0	0	2.3– 9.0	0
VI (11 animals)	0	0	0.6–27.0	0.5–15.6
VII (10 animals)	0	0	0	02.–26.1
VIII (12 animals)	0	0	0	0

* p.d. = pars distalis.

granules. In most of these, however, a PAS-positive substance was present in the lumina of the cell nests. The other grafts contained varying numbers of PAS-positive cells in addition to the PAS-positive substance in the lumina. PAS-positive substance in the lumina was found in normal adult adenohypophyses and in some of the pars distalis fragments, but not in the pituitaries of the newborn. In the graft in which alternate sections were stained with AF, no AF-positive cells were found while PAS-positive cells were present. Some differentiation had occurred in the grafts in comparing them with the pituitaries of the newborn. The amount of growth in the grafts was disappointing, however, as only 6 grafts had a volume of 100% or over of the volume of the newborn pars distalis and none attained the size of the adult adenohypophysis.

SUMMARY

Pituitaries from newborn hamsters when transplanted to the cheek pouch of adult females were unable to support estrus or estrous cycles in the absence of pituitary fragments remaining in the hypophyseal fossae of the hypophysectomized hosts. This confirms the evidence from other mammals that proximity of the adenohypophysis to the hypothalamus is essential for estrous cycles. There was some evidence that the grafts were producing gonadotrophic substances in that removal of the grafts altered the vaginal smear cycles in certain of the animals with pituitary remnants.

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THE ENDOCRINE SOCIETY

1958 ANNUAL MEETING

The Fortieth Annual Meeting of The Endocrine Society will be held in the St. Francis Hotel, San Francisco, California, Thursday, Friday and Saturday, June 19, 20, 21, 1958.

The Committee on Local Arrangements is Dr. Roberto Escamilla, Chairman, Dr. Donald E. Bernstein, Dr. Peter H. Forsham, Dr. Minnie B. Goldberg, Dr. Gilbert S. Gordan, Dr. Francis S. Greenspan, Dr. C. H. Li as members of the committee.

All Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily in the Colonial Ballroom, and in addition there will be simultaneous afternoon sessions in the Italian Room. The annual dinner is scheduled for Friday, June 20th at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately, inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the St. Francis, advising them of time of arrival and departure date. Make your reservations now and avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 15, 1958 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Rulon W. Rawson, 444 E. 68th St., New York 21, New York, not later than February 1, 1958. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.

2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words;
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are

introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

4. Abstracts should be letter perfect, since there will be no opportunity for proofreading by the authors.

NOTICE OF OMISSION

Reference to the paper "On the Progestational Activity of 17 a-Ethynyl-17-hydroxy-5 (10)-estren-3-one (Norethynodrel) by Francis J. Saunders, Richard A. Edgren and Victor A. Drill which appeared in ENDOCRINOLOGY, Volume 60, pages 804–805 was omitted from the subject index and author index of Volume 60 and was not included in the Table of Contents on page VII.